

Simple Method for Markerless Gene Deletion in Multidrug-Resistant *Acinetobacter baumannii*

Man Hwan Oh,^a Je Chul Lee,^b Jungmin Kim,^b Chul Hee Choi,^c Kyudong Han^a

Department of Nanobiomedical Science and BK21 PLUS NBM Global Research Center for Regenerative Medicine, Dankook University, Cheonan, Republic of Korea^a; Department of Microbiology, Kyungpook National University, School of Medicine, Daegu, Republic of Korea^b; Department of Microbiology and Research Institute for Medical Sciences, College of Medicine, Chungnam National University, Daejeon, Republic of Korea^c

The traditional markerless gene deletion technique based on overlap extension PCR has been used for generating gene deletions in multidrug-resistant *Acinetobacter baumannii*. However, the method is time-consuming because it requires restriction digestion of the PCR products in DNA cloning and the construction of new vectors containing a suitable antibiotic resistance cassette for the selection of *A. baumannii* merodiploids. Moreover, the availability of restriction sites and the selection of recombinant bacteria harboring the desired chimeric plasmid are limited, making the construction of a chimeric plasmid more difficult. We describe a rapid and easy cloning method for markerless gene deletion in *A. baumannii*, which has no limitation in the availability of restriction sites and allows for easy selection of the clones carrying the desired chimeric plasmid. Notably, it is not necessary to construct new vectors in our method. This method utilizes direct cloning of blunt-end DNA fragments, in which upstream and downstream regions of the target gene are fused with an antibiotic resistance cassette via overlap extension PCR and are inserted into a blunt-end suicide vector developed for blunt-end cloning. Importantly, the antibiotic resistance cassette is placed outside the downstream region in order to enable easy selection of the recombinants carrying the desired plasmid, to eliminate the antibiotic resistance cassette via homologous recombination, and to avoid the necessity of constructing new vectors. This strategy was successfully applied to functional analysis of the genes associated with iron acquisition by *A. baumannii* ATCC 19606 and to *ompA* gene deletion in other *A. baumannii* strains. Consequently, the proposed method is invaluable for markerless gene deletion in multidrug-resistant *A. baumannii*.

Acinetobacter baumannii is a major opportunistic pathogen causing nosocomial infections in both community and hospital settings (1, 2). *A. baumannii* infections cause serious diseases in immunocompromised human hosts, including pneumonia, bacteremia, sepsis, and meningitis (3). The increase in the resistance of the bacterium to major antimicrobial drugs has become a major worldwide concern (4). However, the pathogenesis of this bacterium has been poorly characterized despite its clinical significance.

The increasing availability of bacterial genome sequences and genomewide association tools, such as transcriptome and comparative genome analysis, have led to the identification of various genes that play important roles in the pathogenesis of *A. baumannii* (5–7). This circumstance has given rise to the need for fast and efficient gene disruption systems, which are critical tools for the functional analysis of genes. The insertion of antibiotic resistance markers into targeted genes using suicide vectors, such as pEX100T and pJQ200, has been widely used for the disruption of *A. baumannii* genes (8–10). Gene replacement using a linear PCR fragment carrying an antibiotic resistance cassette was developed for *A. baumannii* (11). However, these methods result in serious problems with respect to complementation of the mutant and the construction of double gene knockout mutants, especially in multidrug-resistant *A. baumannii* strains. Hence, a method for markerless gene deletion is absolutely essential for the construction of *A. baumannii* mutants.

To date, a cloning method based on overlap extension PCR has been widely used to introduce gene deletions into *A. baumannii* (12, 13). This system involves a procedure in which the downstream and upstream regions of the target gene to be deleted are combined by using overlap extension PCR with locus-specific

primers containing the recognition sites of restriction enzymes. The PCR product digested with the restriction enzymes is cloned into a suicide vector digested with the same restriction enzymes. The chimeric plasmid is integrated within the target sequence via homologous recombination and is then excised by a second single-crossover homologous-recombination event, resulting in allelic exchange. However, this method can be limited by the availability of restriction sites in the cloning steps. Moreover, the insertion of an unmarked DNA fragment into the plasmid results in problems such as a high background of false-positive colonies (without the desired chimeric plasmid) caused by the self-ligation of digested plasmids. Notably, the construction of new vectors containing a suitable antibiotic resistance cassette is required for the construction of mutants of clinical isolates that exhibit different profiles of resistance to a variety of antibiotics. Therefore, this method is time-consuming and inefficient for the construction of mutants of multidrug-resistant *A. baumannii*.

Recently, Tucker et al. (14) developed a method based on Re-

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Address correspondence to Chul Hee Choi, choich@cnu.ac.kr, or Kyudong Han, jim97@dankook.ac.kr.

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cET recombinase and flippase (FLP)/FLP recognition target (FRT) sites for markerless gene deletion in *A. baumannii*. This method generates PCR products by using primers with 100- to 125-nucleotide extensions that are homologous to regions adjacent to the gene to be disrupted and a template plasmid carrying an antibiotic resistance cassette that is flanked by FRT sites. The method utilizes RecET recombinase and FLP/FRT systems for the recombination and elimination of the antibiotic resistance cassette, respectively. Consequently, two different antibiotic resistance markers, one for the template plasmid and one for the RecET/FLP system, are absolutely necessary for the method. Hence, its application is inevitably restricted for multidrug-resistant clinical isolates of *A. baumannii*, since available antibiotic resistance markers are extremely limited.

In this study, we describe a new method for markerless gene deletion that is based on a rapid and simple cloning strategy using direct insertion of a blunt-end PCR product into a blunt-end suicide vector. Briefly, the blunt-end DNA fragments containing upstream and downstream regions of the target gene and an antibiotic resistance cassette are sequentially assembled via overlap extension PCR and are then directly cloned into the blunt-end suicide vector developed in this study. The recombinant bacteria carrying the desired chimeric plasmid are easily selected on a medium containing the antibiotic that corresponds to the antibiotic resistance cassette inserted outside the downstream region of the target gene. The plasmid-borne deletion allele with the antibiotic resistance cassette is integrated into the targeted sequence by using a conjugation-based gene transfer system and homologous recombination. *A. baumannii* merodiploids are selected on the medium supplemented with the antibiotic corresponding to the antibiotic resistance cassette inserted outside the downstream region of the target gene. Importantly, the plasmid and antibiotic resistance cassette are simultaneously eliminated via a second single-crossover homologous-recombination event by sucrose counter-selection, resulting in allelic exchange. In the present study, the effectiveness of the proposed method was demonstrated by deletion of *basD* and *bauA*, which are essential for the survival of *A. baumannii* ATCC 19606 under iron-depleted conditions (15, 16). The proposed method was also applied to the functional analysis of genes in siderophore gene cluster 1 of *A. baumannii* ATCC 19606. Finally, we chose two different clinical isolates of *A. baumannii*, ATCC 17978 and 1656-2, with which to investigate the applicability of the proposed method. In order to choose a suitable antibiotic resistance cassette in mutant construction, the antibiotic susceptibilities of *A. baumannii* ATCC 17978 and 1656-2 were examined. Each of the *ompA* genes in these *A. baumannii* strains was successfully deleted.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *A. baumannii* were grown in Luria-Bertani (LB) broth alone or in LB broth containing 1.5% (wt/vol) agar at 37°C. For plasmid maintenance in *E. coli*, chloramphenicol (20 µg/ml), erythromycin (400 µg/ml), or kanamycin (50 µg/ml) was added to the growth medium. After conjugation, *A. baumannii* merodiploids were selected on the medium supplemented with ampicillin (100 µg/ml) and kanamycin (30 µg/ml) or erythromycin (30 µg/ml). All the medium components were purchased from Difco (Detroit, MI, USA), and the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

DNA manipulations. Genomic and plasmid DNAs were purified from bacteria using a SolGent Genomic DNA prep kit (SolGent, Daejeon, Republic of Korea) and an AccuPrep plasmid extraction kit (Bioneer, Daejeon, Republic of Korea), respectively. DNA fragments were purified with an AccuPrep gel purification kit (Bioneer). Restriction and DNA-modifying enzymes were purchased from New England BioLabs (Ipswich, MA, USA). Routine DNA manipulations were performed as described previously (17) or according to the manufacturer's recommendations.

Construction of the pHKD01 vector. For direct cloning of blunt-end PCR products, the multicloning sites of pDS132 were replaced by annealed oligonucleotide cloning. To generate the annealed oligonucleotides, each complementary single-stranded oligonucleotide (Fig. 1) was resuspended in annealing buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, and 1 mM EDTA. The reaction was initiated by mixing equal amounts (2 µg) of each oligonucleotide in a total volume of 50 µl. Annealing conditions were set to 95°C for 5 min and 25°C for 60 min. The annealed oligonucleotides were ligated with pDS132 digested with PstI.

Primers, PCR, and plasmid construction. All oligonucleotides used for mutant construction are listed in Table 2. All primers were designed to yield melting temperatures of 54 to 56°C and PCR fragments of approximately 1,000 to 1,200 bp. The primers were designed to combine the upstream and downstream regions of the coding regions of the target genes with the kanamycin or erythromycin resistance cassette by overlap extension PCR. Reverse primers for amplification of the upstream and downstream sections contained 25 additional nucleotides at their 5' ends that were homologous to the downstream region and the antibiotic resistance cassette, respectively. The genomic DNAs purified from *A. baumannii* strains and pUC4K (or pIL252) for amplification of the kanamycin resistance cassette (or erythromycin resistance cassette) were used as templates for the PCR. PrimeSTAR GXL *Taq* DNA polymerase (TaKaRa, Shiga, Japan), which exhibits 30 mismatched bases per 486,923 total bases and higher fidelity than *Pfu* DNA polymerase, was chosen to prevent errors in the PCR products generated by overlap extension PCR and to produce blunt-end PCR products. PCR was performed using 1.25 U PrimeSTAR GXL *Taq* DNA polymerase, 10 µl of 5× PrimeSTAR GXL buffer, 0.2 mM deoxynucleoside triphosphate (dNTP) mixture, 10 pM of each primer, and template DNA (100 ng). Each mutated fragment was amplified by two-step PCR. For the first step (PCR-1), PCR cycle conditions were as follows: 30 cycles of 98°C for 10 s, 54°C for 40 s, and 68°C for 2 min, with a final elongation at 68°C for 10 min. The products obtained from the first step of PCR were then mixed at equimolar concentrations and were subjected to overlap extension PCR with the forward primers corresponding to the upstream region and the reverse primers corresponding to the antibiotic resistance cassette. PCR cycle conditions were as follows: 30 cycles of 98°C for 10 s, 54°C for 40 s, and 68°C for 4 min, with a final elongation at 68°C for 10 min. The blunt-end PCR products containing each deleted target gene with the antibiotic resistance cassette were ligated with FspI-digested pHKD01 to generate chimeric plasmids (Table 1). The plasmids constructed were confirmed by DNA sequencing.

Conjugation and plasmid curing with an antibiotic resistance cassette. The *E. coli* S17-1 λ pir strains containing each constructed plasmid were used as conjugal donors for *A. baumannii* strains. The transconjugants were conjugated and isolated as follows. Donor and recipient strains were grown in LB broth until the late-log phase (optical density at 600 nm [OD₆₀₀], 0.8). The bacterial cells were then mixed at an equal ratio and were spotted onto an LB plate. The bacterial cells were incubated for 12 h at 30°C. The bacteria were resuspended in LB broth and were then plated onto LB agar plates containing ampicillin and kanamycin or erythromycin in order to eliminate the donor strains and select the merodiploid strains. Plasmids with the antibiotic resistance cassette were excised from the bacterial chromosome on solid LB medium with 10% sucrose and without NaCl (13). Each mutant was confirmed by PCR analysis.

qRT-PCR and survival under iron-depleted conditions. *A. baumannii* was grown in an iron-replete medium, LB medium. LB medium with 2,2'-dipyridyl (DIP) added to a final concentration of 0.2 mM was used as

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Bacterial strains		
<i>A. baumannii</i>		
ATCC 19606	Prototype strain	26
ATCC 17978	Prototype strain	27
1656-2	Clinical isolate	18
HKD01	ATCC 19606 with Δ <i>basD</i>	This study
HKD02	ATCC 19606 with Δ <i>bauA</i>	This study
HKD03	ATCC 19606 with Δ <i>rimL</i>	This study
HKD04	ATCC 19606 with Δ <i>piuB</i>	This study
HKD05	ATCC 19606 with Δ <i>cirA</i>	This study
HKD06	ATCC 19606 with Δ <i>menG</i>	This study
HKD07	ATCC 19606 with Δ <i>fluF</i>	This study
HKD08	ATCC 19606 with Δ <i>rhcC1</i>	This study
HKD09	ATCC 19606 with Δ <i>rhcC2</i>	This study
HKD10	ATCC 19606 with Δ <i>araJ</i>	This study
HKD11	ATCC 19606 with Δ <i>iucD</i>	This study
HKD12	ATCC 19606 with Δ <i>rhcC3</i>	This study
HKD13	HKD02 with Δ <i>cirA</i>	This study
HKD14	ATCC 17978 with Δ <i>ompA</i>	This study
HKD15	1656-2 with Δ <i>ompA</i>	This study
<i>E. coli</i>		
DH5 α λ pir	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> λ pir (phage lysogen); plasmid replication	Laboratory collection
S17-1 λ pir	λ pir lysogen; <i>thi pro hsdR hsdM⁺ recA</i> RP4-2 Tc::Mu-Km::Tn7; Tp ^f Sm ^r ; host for π -requiring plasmids; conjugal donor	28
Plasmids		
pDS132	Suicide vector; <i>oriR6K sacB</i> ; Cm ^r	19
pUC4K	pUC4 with <i>nptI</i> ; Ap ^r Km ^r	Pharmacia
pIL252	Em ^r ; pAM β 1 <i>ori</i>	29
pHKD01	pDS132, multicloning sites; <i>oriR6K sacB</i> ; Cm ^r	This study
pHKD02	pHKD01 with Δ <i>basD</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD03	pHKD01 with Δ <i>bauA</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD04	pHKD01 with Δ <i>rimL</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD05	pHKD01 with Δ <i>piuB</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD06	pHKD01 with Δ <i>cirA</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD07	pHKD01 with Δ <i>menG</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD08	pHKD01 with Δ <i>fluF</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD09	pHKD01 with Δ <i>rhcC1</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD10	pHKD01 with Δ <i>rhcC2</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD11	pHKD01 with Δ <i>araJ</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD12	pHKD01 with Δ <i>iucD</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD13	pHKD01 with Δ <i>rhcC3</i> :: <i>nptI</i> ; Cm ^r Km ^r	This study
pHKD14	pHKD01 with ATCC 17978 Δ <i>ompA</i> :: <i>nptI</i> ; Cm ^r Em ^r	This study
pHKD15	pHKD01 with 1656-2 Δ <i>ompA</i> :: <i>nptI</i> ; Cm ^r Em ^r	This study

^a Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Km^r, kanamycin resistant.

an iron-depleted medium. Total RNAs from *A. baumannii* grown to an OD₆₀₀ of 0.7 were isolated by using an RNeasy minikit (Qiagen, Valencia, CA, USA). For quantitative real-time PCR (qRT-PCR), cDNA was synthesized by using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), and real-time PCR amplification of the cDNA was carried out in a Chromo4 real-time PCR detection system (Bio-Rad Laboratories) with a pair of specific primers, as listed in Table 3. The relative expression levels of the targeted genes were calculated by using the 16S rRNA expression level as the internal control for normalization.

The survival of *A. baumannii* under iron-depleted conditions was investigated by measuring the growth of the bacteria cultured in the iron-depleted LB medium. *A. baumannii* was grown in LB medium overnight at 37°C. The overnight cultures of *A. baumannii* were washed using phosphate-buffered saline (pH 7.4). One hundred microliters of the cultures was inoculated into 100 ml of the iron-depleted LB medium and was

shaken at 37°C. After 10 h of culture, the bacterial growth was monitored by measuring the OD₆₀₀ of the cultures.

Biofilm assay. Biofilms formed by *A. baumannii* were measured using a gentian violet staining assay (18). Briefly, overnight cultures were adjusted to an OD₆₀₀ of 2.0 and were diluted 200-fold in LB medium. Aliquots (1 ml) of the bacterial suspension were then transferred to 5-ml polystyrene tubes. Biofilms were formed by incubating these cultures for 14 h at 37°C without shaking. Planktonic cells were removed, and the tubes were then washed twice with 1 ml of phosphate-buffered saline (pH 7.4). The biofilm cells on the wall were stained with 0.1% (wt/vol) gentian violet solution for 15 min at room temperature. The biofilms were quantitated by measuring the amount of gentian violet eluted from the biofilms as the OD₅₇₀ normalized to total bacterial growth (OD₆₀₀).

Data analysis and statistics. Averages and standard errors of the means (SEM) were calculated from at least three independent experi-

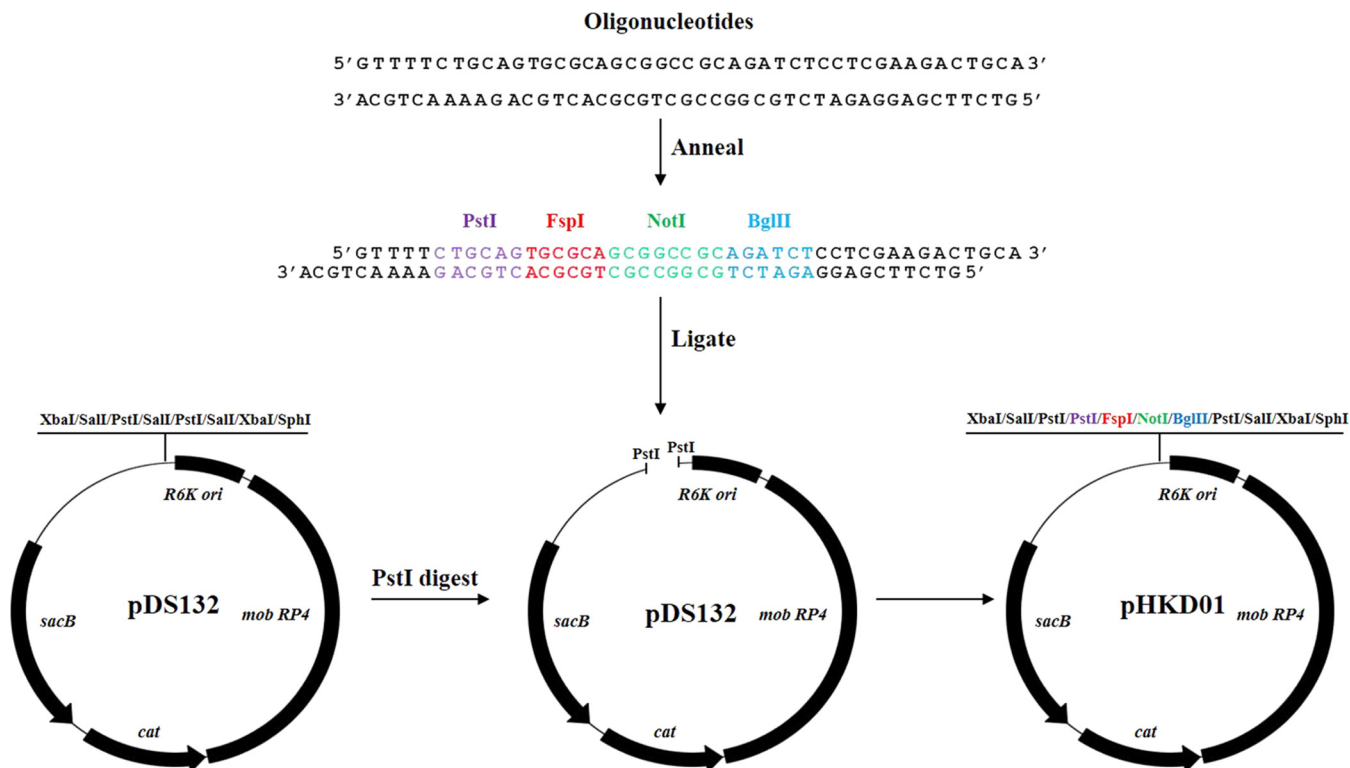


FIG 1 Construction of pHKD01. Complementary oligonucleotides were designed to generate restriction enzyme sites for PstI, FspI, NotI, and BglII. Annealing between the oligonucleotides was performed as described in Materials and Methods. The annealed oligonucleotides were ligated with PstI-digested pDS132. The added restriction enzyme sites are indicated by different colors. *sacB*, levansucrase-encoding gene; *R6K ori*, replication origin; *cat*, chloramphenicol resistance gene; *mob RP4*, plasmid mobilization region.

ments. All data were analyzed by Student *t* tests with SAS software (SAS Institute Inc., Cary, NC, USA). Differences between experimental groups were considered significant at a *P* value of <0.005.

RESULTS

pHKD01 construction for direct cloning of blunt-end PCR products. pDS132, a pCVD442 derivative in which the *IS1* element was removed to prevent untargeted integration of the plasmid into bacterial chromosomes, has been widely used for gene allele exchange in bacteria (19). However, only four restriction enzyme sites for XbaI, SalI, PstI, and SphI exist in the multicloning sites of pDS132. The restricted availability of multicloning sites causes difficulties during the cloning step. Therefore, we constructed a pDS132 derivative carrying alternative cloning sites for direct cloning of blunt-end PCR products. The construction of pHKD01 is presented in Fig. 1. The multicloning sites of pDS132 were replaced via annealed oligonucleotide cloning. We designed complementary oligonucleotides (Fig. 1), in which the recognition sites for PstI, FspI, NotI, and BglII exist consecutively, to be cloned directly into the overhangs of the pDS132 plasmid generated by PstI digestion. Especially, the FspI restriction site was chosen to produce a blunt-end vector. The annealed oligonucleotides were ligated with the PstI-digested pDS132. Correct plasmid construction was confirmed by DNA sequencing. The new suicide plasmid was named pHKD01.

Markerless deletion of the *basD* and *bauA* genes in *A. baumannii* ATCC 19606. The principle for the markerless gene deletion method is diagrammed in Fig. 2. The amplification of mutated

DNA fragments is accomplished in two PCR steps (PCR-1 and PCR-2) with six PCR primers. Four of these are unique to the upstream and downstream regions (approximately 1 kb each) of the coding region of the target gene to be deleted. Two of these are common primers for the amplification of *npfI*, conferring kanamycin resistance. For example, the upstream and downstream regions of target gene X are amplified from the genomic DNA of *A. baumannii* using primer pairs S1/S2 and S3/S4 (Fig. 2), respectively. The kanamycin resistance cassette is amplified using primer pair U1/U2 (Table 2) with pUC4K as the template. In particular, primers S2 and S4 contain 25 nucleotides at their 5' ends that are homologous to the downstream region of the target gene and the kanamycin resistance cassette, respectively. The three PCR products obtained in the first step are mixed at equimolar concentrations and are subjected to overlap extension PCR with primers S1 and U2 (Fig. 2). The mutated DNA fragment generated by overlap extension PCR is ligated directly into the blunt-end plasmid pHKD01, which was created by digestion with FspI. The plasmid is integrated into the chromosome of *A. baumannii* by using conjugation-based gene transfer and homologous recombination. In the first single-crossover homologous-recombination event, merodiploids that possess an integrated copy of pHKD01 containing the mutated DNA fragment are readily obtained on LB agar plates containing kanamycin and ampicillin. The second single-crossover homologous-recombination event in the bacteria is achieved with *sacB*, conferring sucrose sensitivity, on LB agar plates containing sucrose. This step allows the bacterial cells to

TABLE 2 Oligonucleotides used for the construction of mutants

Oligonucleotide function and designation	Oligonucleotide sequence (5' → 3') ^a	Target gene	Locus tag
Gene deletion in <i>A. baumannii</i> ATCC 19606			
BASD01F	GAAGCAATTGAGCGGTTCCAGG	<i>basD</i>	HMPREF0010_02303
BASD01R	<u>AAATGATGAAAAGTTCAAAAATACGAA</u> TTGTTAATCATTTCCAATTTTGCTGT		
BASD02F	TTCGTATTTTGAACCTTTCATCATTTTGG		
BASD02R	<u>GCAACACCTTCTTCACGAGGCAGACT</u> GTGCAAAACAGGTTAAACGCAG		
BAUA01F	GTGCGATTTGGAGAAAACGTAAGC	<i>bauA</i>	HMPREF0010_02301
BAUA01R	<u>CATAAAATTTTCACATTTTGTGCTATG</u> ATAAAAAAACCCGCAATCGC		
BAUA02F	CATAGCAAAATAGTGAAAATTTATGCTC		
BAUA02R	<u>GCAACACCTTCTTCACGAGGCAGACAT</u> CATAGCGGCAGCTGTCC		
RIML01F	ACTTTGCTTCTATCTGGATGCG	<i>rimL</i>	HMPREF0010_00723
RIML01R	<u>CTTGCAGGTCTACCCATACCGAAAA</u> CGGTCTTGTCTATGACATAACC		
RIML02F	TTTCTGGTATGGGTAGACCTGC		
RIML02R	<u>GCAACACCTTCTTCACGAGGCAGAC</u> CGGGATAAATTGATCAAGATCGG		
PIUB01F	GGCTTAAATCTGGGATTGACTGG	<i>piuB</i>	HMPREF0010_00725
PIUB01R	<u>CCCAAAAATAATGATGCAAAAGCTCA</u> TTTAAAGCCACTCCCCATTAGCTA		
PIUB02F	ATGAGCTTTGCATCATTATTTTGG		
PIUB02R	<u>GCAACACCTTCTTCACGAGGCAGAC</u> GCACATGTCTTCTACTACAGCCG		
CIRA01F	AGTTAGCGAAAATAGGTCGGTACC	<i>cirA</i>	HMPREF0010_00727
CIRA01R	<u>ACAGGCATCTCTTTTCGTTAGTTGC</u> ATTCTCCCTAGCCCAAATGTTACTCAAC		
CIRA02F	TGCAACTAACGAAAGAGATGCCTG		
CIRA02R	<u>GCAACACCTTCTTCACGAGGCAGACA</u> ACAGTCCAGTAAAGGCCATCACC		
MENG01F	TGGTCTGTACTTGTCTATGCGT	<i>menG</i>	HMPREF0010_00728
MENG01R	<u>CGAACATTAATGAGAATGATTTTACT</u> TTTAAATTTCCGATTCTTTTAAACGTAAG		
MENG02F	TAAAAATCATTCTCAATTAATGTTGGTATT		
MENG02R	<u>GCAACACCTTCTTCACGAGGCAGAC</u> GAGTAATCAGGTCGGTAGTCAGTATCC		
FHUF01F	CCGATAGGAATTAAGCTCTTGG	<i>fhuF</i>	HMPREF0010_00729
FHUF01R	<u>CITTAGAAGTATCAATTTGATTCA</u> TCGTCAGATATCACTAACAAATTAAGGC		
FHUF02F	ATGAATCAAATGATACTTCTAAAGCACT		
FHUF02R	<u>GCAACACCTTCTTCACGAGGCAGACA</u> GCAACTTACGACCTGCCG		
RHBC101F	GCCCAACAATCTCTACGCAC	<i>rhbC1</i>	HMPREF0010_00730
RHBC101R	<u>AAACATATTTCATCGTCAGATATCA</u> AGATAAATCTCACTTCACCTGCAAT		
RHBC102F	TGATATCTGACGATGAATATGCTTTTAA		
RHBC102R	<u>GCAACACCTTCTTCACGAGGCAGAC</u> GACTCAATCCGAACCGTAGC		
RHBC201F	CCATGATCATGCTAACCTTAGCAG	<i>rhbC2</i>	HMPREF0010_00731
RHBC201R	<u>GCTGAAGTGCATTCATAGATAAAAT</u> CTTTTAAATCCTATTTCTTTAATTGGTGC		
RHBC202F	GATTTATCTATGAATGCACCTTCAGCC		
RHBC202R	<u>GCAACACCTTCTTCACGAGGCAGAC</u> CCAAAGAGCTTTAATTCCTATCGG		
ARAJ01F	GGGTACAACCTCCACATTTACCAG	<i>araJ</i>	HMPREF0010_00732
ARAJ01R	<u>TGTAATTGTCCTATTTTATTAATCA</u> TCCTCTGTTAGCTTATTCATATGCG		
ARAJ02F	GATTAATAAAAAATGGGCAATTCATCC		
ARAJ02R	<u>GCAACACCTTCTTCACGAGGCAGACA</u> CCAAACAGCTGTGAATAAATGGC		
IUCD01F	GATATTGGTTTACGTGGATGGC	<i>iucD</i>	HMPREF0010_00733
IUCD01R	<u>TCCATACACATATCCTCTGTTT</u> AGCTCAAGCATAACTCACATCCTTCTGT		
IUCD02F	GCTAAACAGAGGATATGTGTATGGAAC		
IUCD02R	<u>GCAACACCTTCTTCACGAGGCAGACA</u> TCATTAGCGCAAAACAGTCC		
RHBC301F	ATTGTTTTTTGTTGCGGTGTTCCG	<i>rhbC3</i>	HMPREF0010_00734
RHBC301R	<u>GTCCAATFCCAATAAAATCAAGCA</u> TCGACTACATCCTATTCAGAATCTTAAGC		
RHBC302F	ATGCTTGATTTTATTGGAATTGGGA		
RHBC302R	<u>GCAACACCTTCTTCACGAGGCAGAC</u> TGCTGCAATAACACAATCAGCC		
Gene deletion in <i>A. baumannii</i> ATCC 17978			
OMPA101F	ATGTCAGTCATTGTTGTAATCTCCG	<i>ompA</i>	A1S_2840
OMPA101R	<u>CITTTTTACTGTTCAAGAACTCAA</u> AGTGCAATACGACTC AATTTTCATGG		
OMPA102F	TGAGTTCTTGAACAGTAAAAAAGCG		
OMPA102R	<u>ATGGTGAAGTCAGCAGCAACACG</u> AGGCTTGTCCGAATGCTTCC		
Gene deletion in <i>A. baumannii</i> 1656-2			
OMPA201F	TGACTTCTTCGACATCTGTAGGC	<i>ompA</i>	ABK1_3141
OMPA201R	<u>TCGCTTTTTACTGTTCAAGAACT</u> CGGATATCCTCCAGAG ATAACAATTG		
OMPA202F	GAGTTCCTTGAACAGTAAAAAAGCGAC		
OMPA202R	<u>ATGGTGAAGTCAGCAGCAACACG</u> ACCATTGAACGAAAAGTGCC		
Amplification of the kanamycin resistance cassette			
U1	GTCTGCCTCGTGAAGAAGGTG	<i>nptI</i>	
U2	GATCCGTCGACCTGCAGG		
Amplification of the erythromycin resistance cassette			
U3	TCGTGTTGCTGCTGACTTGC	<i>ermAM</i>	
U4	GACCTCTTGTAGCTCCTTGGAAAGC		

^a The oligonucleotides were designed using the genome sequences of *A. baumannii* ATCC 19606 (GenBank accession number [ACQB00000000](#)), ATCC 17978 (GenBank accession number [CP000521](#)), and 1656-2 (GenBank accession number [CP001921](#)). Regions of oligonucleotides not complementary to the corresponding templates are underlined.

TABLE 3 Oligonucleotides used for quantitative real-time PCR

Oligonucleotide	Oligonucleotide sequence (5' → 3')	Target gene
RIML03F	GCTACTCATTACGTCAGGTTTCAG	<i>rimL</i>
RIML03R	CCACTGAGGAATAACGTGTGG	
PIUB03F	AAAGATGGCGAGTCACAGTAG	<i>piuB</i>
PIUB03R	CCCAATAGCTCTCCGGTATTAG	
CIRA03F	GTAGTAAGTGCAGCTCGTACAC	<i>cirA</i>
CIRA03R	GCCGTAGCTTGCTGGATAA	
MENG03F	CGTTGTGGTCTTAGGTGCTATT	<i>menG</i>
MENG03R	CCCTTCATTGCGAGTGGTTA	
FHUF03F	GTGCGTTTATGGAATGGATGG	<i>fluF</i>
FHUF03R	AGGGACAATTACGGCATAAGG	
RHBC103F	CAAAGCTTGGCACATAGATTCC	<i>rhbC1</i>
RHBC103R	CTTGCTGACTGAGACCTTCTT	
RHBC203F	GGCAAAGTCAGGATCGCTATT	<i>rhbC2</i>
RHBC203R	ACGTGAGCTTGATGTGTTAGTT	
ARAJ03F	CATGGCTTAGGAATGGGACTT	<i>araJ</i>
ARAJ03R	GGCCGCTTGAAGTAAGTAGATG	
IUCD03F	TGATGTGGAGGTAGTGTGAATG	<i>iucD</i>
IUCD03R	GCTTTCTGGTAAATGTGGAGTTG	
RHBC303F	GAGCAGCCAATTTTCAGATCAAG	<i>rhbC3</i>
RHBC303R	GAGCTTGCCAAGGATGTAGT	

excise the plasmid with *nptI*. The bacteria sensitive to kanamycin are selected, and the target gene deletion is then confirmed by PCR analysis.

To validate the proposed method, *basD* and *bauA*, required for iron acquisition by *A. baumannii* (15, 16), were selected as the target genes to be deleted in *A. baumannii* ATCC 19606. In PCR-1, the upstream and downstream regions of each target gene and *nptI* were amplified by PCR with each locus-specific primer as indicated in Table 2 (Fig. 3A). In PCR-2, the approximately 3.2-kb DNA fragments, in which the upstream and downstream regions and *nptI* were combined by overlap extension PCR (Fig. 3A), were gel purified and cloned into *FspI*-digested pHKD01 to yield pHKD02 ($\Delta basD::nptI$) and pHKD03 ($\Delta bauA::nptI$) (Table 1). *E. coli* S17-1 *λpir* containing each of the resulting plasmids was used as a conjugal donor to generate the isogenic mutants of *A. baumannii* ATCC 19606 by homologous recombination. As shown in Fig. 3B, a second single-crossover homologous-recombination event, by which each target gene was deleted, was confirmed by PCR. PCR analysis of the genomic DNA of the wild type using primers BASD01F and BASD02R produced a 4.9-kb fragment, whereas the genomic DNA from the *basD* mutant HKD01 resulted in an amplified DNA fragment of approximately 2.0 kb (Fig. 3B). The size of this 2.0-kb fragment was in good agreement with the projected size of the DNA fragment without *basD* (2.9 kb). In addition, identification of the mutant in which *bauA* was deleted was confirmed by PCR using primers BAUA01F and BAUA02R. PCR analysis of the genomic DNA of the wild type and of *bauA* mutant HKD02 indicated fragment sizes of 4.3 kb and 2.0 kb, respectively (Fig. 3B). Therefore, the combined results demonstrated that the *basD* and *bauA* genes were successfully deleted in the HKD01 and HKD02 strains, respectively. Furthermore, to examine the effects of *basD* and *bauA* mutation on the survival of *A. baumannii* under iron-depleted conditions, the growth of each mutant strain under iron-depleted conditions was compared to that of the parental wild-type strain (Fig. 3C). Under iron-replete conditions (LB medium), the growth of the mutants did not differ from that of the wild type. However, the growth of the mutant

strains was significantly impaired when they were cultured in iron-depleted LB medium. This is consistent with the previous observation that BasD and BauA are essential for the survival of *A. baumannii* under iron-depleted conditions (15).

Functional analysis of siderophore gene cluster 1 in *A. baumannii* ATCC 19606 through markerless gene deletion. *A. baumannii* strains have acquired different siderophore gene clusters (20). *A. baumannii* ATCC 19606 has siderophore gene cluster 1 on the bacterial genome. However, the functional aspects of these genes within the gene cluster have not been characterized. Therefore, the proposed method was used to analyze their roles in bacterial growth under iron-depleted conditions. Ten genes (*rimL*, *piuB*, *cirA*, *menG*, *fluF*, *rhbC1*, *rhbC2*, *araJ*, *iucD*, and *rhbC3*) within the gene cluster (Fig. 4A) were selected as the target genes for deletion. Before proceeding with mutant construction, we investigated whether the expression of these genes is regulated by the level of iron. The expression levels of the genes in *A. baumannii* ATCC 19606 grown in either iron-depleted LB medium (with 0.2 mM DIP) or LB medium alone were analyzed by qRT-PCR (Fig. 4B). The expression levels of *rimL*, *piuB*, *cirA*, *menG*, *fluF*, *rhbC1*, *rhbC2*, *araJ*, *iucD*, and *rhbC3* in bacteria grown in the iron-depleted medium were significantly increased 196 (\log_{10} 2.29)-, 10 (\log_{10} 1.00)-, 27 (\log_{10} 1.43)-, 3 (\log_{10} 0.41)-, 11 (\log_{10} 1.02)-, 105 (\log_{10} 2.02)-, 16 (\log_{10} 1.20)-, 26 (\log_{10} 1.41)-, 28 (\log_{10} 1.45)-, and 95 (\log_{10} 1.98)-fold, respectively. These results indicated that each target gene is regulated by the level of iron in the growth medium. Hence, the 10 genes were inactivated separately by using the overlap extension PCR method proposed in this study. The disrupted genes were cloned into pHKD01 to result in pHKD04 ($\Delta rimL::nptI$), pHKD05 ($\Delta piuB::nptI$), pHKD06 ($\Delta cirA::nptI$), pHKD07 ($\Delta menG::nptI$), pHKD08 ($\Delta fluF::nptI$), pHKD09 ($\Delta rhbC1::nptI$), pHKD10 ($\Delta rhbC2::nptI$), pHKD11 ($\Delta araJ::nptI$), pHKD12 ($\Delta iucD::nptI$), and pHKD13 ($\Delta rhbC3::nptI$) (Table 1). Each of the genes was deleted using a conjugation-based gene transfer system and homologous recombination. Each mutant was confirmed by PCR using a pair of primers as indicated in Table 2 (Fig. 4C), and mutants were named HKD03, HKD04, HKD05, HKD06, HKD07, HKD08, HKD09, HKD10, HKD11, and HKD12 (Table 1). Moreover, to evaluate the efficiency of the proposed method in markerless gene deletion, PCR analysis was performed to examine the absence of the kanamycin resistance cassette in the bacterial strains obtained through sucrose treatment. Approximately 98% of the colonies were confirmed to be sucrose-resistant colonies in which the antibiotic resistance cassette with pHKD01 was eliminated by a second single-crossover homologous-recombination event (Table 4). The sucrose-resistant colonies were also used to evaluate the success rate of deletion of the target genes by using PCR analysis. The average success rate was approximately 48%, but for individual target genes, the success rate of deletion differed (20% to 77%, approximately) (Table 4).

To examine the effects of the mutated genes on survival under iron-depleted conditions, the growth of the mutant strains cultured in either iron-replete or iron-depleted medium was compared with that of the wild-type strain. As shown in Fig. 4D, no difference in growth was observed among bacterial strains cultured in LB medium. However, the growth of the *rimL* mutant strain was significantly decreased in the iron-depleted medium, whereas the growth of other mutant strains was comparable to that of the wild type. These results indicated that RimL is likely to

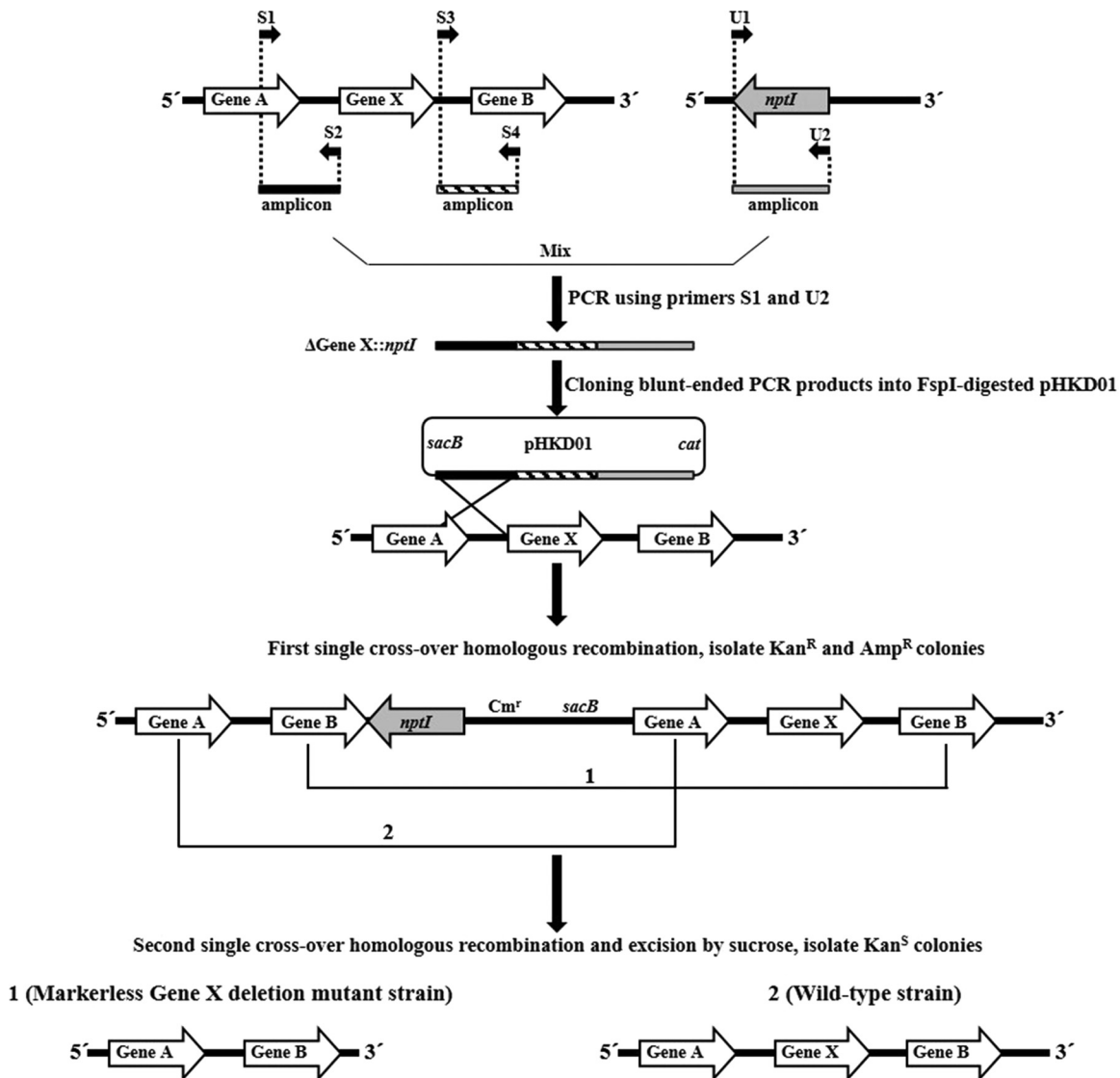


FIG 2 Schematic representation of markerless gene deletion based on rapid and simple cloning. The first step consists of independently amplifying the upstream and downstream regions of target gene X (to be deleted) and *nptI* (conferring kanamycin resistance) by using primer sets S1/S2, S3/S4, and U1/U2, respectively. The upstream and downstream regions of gene X and *nptI* are sequentially assembled by overlap extension PCR with primers S1 and U2. The blunt-end DNA fragments are directly cloned into FspI-digested pHKD01. The plasmid is integrated into the chromosome of *A. baumannii* by conjugation and homologous recombination. The excision of the plasmid region with *nptI* in the chromosome via a second single-crossover homologous-recombination event, by sucrose counterselection, on the side of the region opposite that of the first integration event leads to markerless gene deletion. The markerless deletion mutant is confirmed by PCR. *sacB*, levansucrase-encoding gene; *cat*, chloramphenicol resistance gene; Kan^r, kanamycin resistant; Amp^r, ampicillin resistant; Kan^s, kanamycin sensitive.

be essential for the survival of *A. baumannii* ATCC 19606 under iron-depleted conditions.

Double gene deletion in *A. baumannii* ATCC 19606. To investigate the effectiveness of the proposed method in double gene deletion, we chose *cirA* as a target gene to be deleted in a single gene deletion mutant (the *bauA* mutant) of *A. baumannii*. To generate double deletion mutants, pHKD06 (Δ *cirA*::*nptI*) was integrated into the chromosome of HKD02 (*bauA* mutant) by conjugative transfer from *E. coli* S17-1 λ pir. The merodiploid strain was selected on LB agar plates containing ampicillin and kanamycin. For excision of the plasmid with the kanamycin resistance cassette, a second single-crossover homologous-recombination event was introduced by sucrose counterselection. The deletion of

the *cirA* gene in the double mutant (HKD13) (Table 1) was confirmed by PCR (Fig. 5). PCR analysis of the genomic DNA from the wild-type strain with primer pairs BAUA01F/BAUA02R and CIRA01F/CIRA02R revealed 4.3-kb fragments, whereas the genomic DNA from HKD13 resulted in amplified DNA fragments of approximately 2.0 kb. These results demonstrated that the markerless gene deletion method suggested in the present study is a useful tool for introducing an additional mutation into *A. baumannii*.

Application of markerless gene deletion to *A. baumannii* strains other than ATCC 19606. To demonstrate the applicability of the proposed method in two different clinical strains, *A. baumannii* ATCC 17978 and 1656-2, which exhibit different pheno-

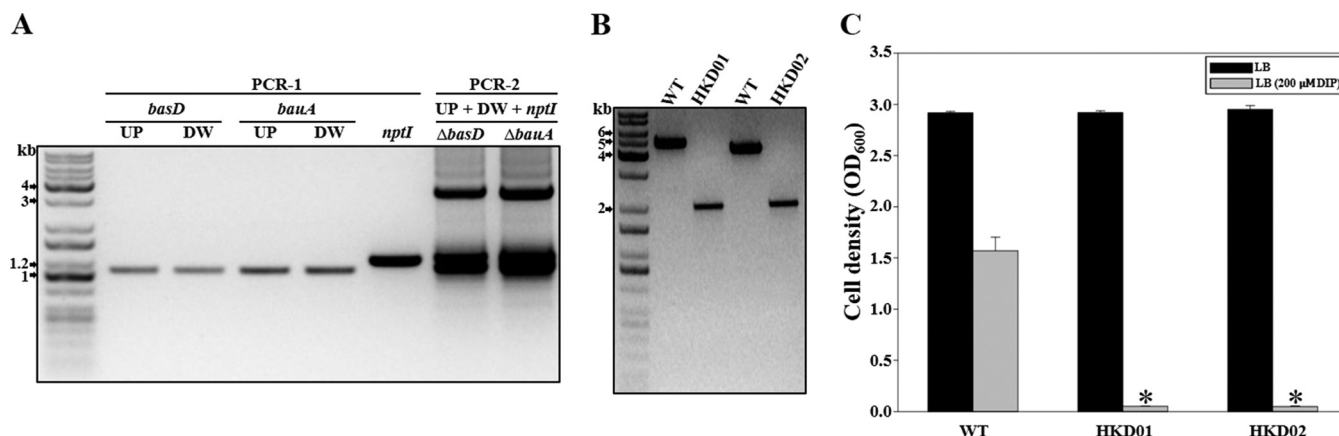


FIG 3 Construction of *basD* and *bauA* mutants. (A) In PCR-1, the downstream and upstream regions of *basD* and *bauA* were amplified by PCR using the genomic DNA of *A. baumannii* ATCC 19606. *nptI*, conferring kanamycin resistance, was also amplified by PCR using pUC4K as a template. In PCR-2, the DNA fragments with the target genes deleted were combined with *nptI* by overlap extension PCR. UP, upstream region of the target gene to be deleted; DW, downstream region of the target gene to be deleted. (B) PCR analysis of the wild-type and mutant strains generated by allelic exchange. Molecular size markers (1-kb DNA ladder; Kapa) are indicated. (C) The growth of the strains on LB medium without and with 0.2 mM DIP was measured after 10 h. Error bars represent SEM. Means and SEM were calculated from three independent experiments. Asterisks indicate significant differences ($P < 0.0001$) from the wild type cultured in LB medium with 0.2 mM DIP. WT, wild type; HKD01, *basD* mutant; HKD02, *bauA* mutant.

types with regard to antibiotic resistance and biofilm formation (18, 21), were selected. In order to choose a suitable antibiotic resistance cassette for the construction of mutants of these strains, the susceptibilities of these strains to chloramphenicol, kanamycin, and erythromycin were investigated by monitoring their growth on LB medium supplemented with different concentrations of these antibiotics (Fig. 6A). The growth of *A. baumannii* ATCC 17978 was not effectively suppressed by the addition of 30 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$ of chloramphenicol to the medium. However, the growth of *A. baumannii* ATCC 17978 was significantly impaired in the presence of kanamycin (30 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$) or erythromycin (30 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$). On the other hand, the growth of *A. baumannii* 1656-2 was substantially inhibited only by erythromycin (30 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$). Therefore, for *A. baumannii* ATCC 17978 and 1656-2, an erythromycin resistance cassette was used as a selective marker for the construction of chimeric plasmids and the selection of their merodiploids.

The *ompA* gene, essential for biofilm formation by *A. baumannii* ATCC 19606 (22), was chosen as the target gene to be deleted in *A. baumannii* ATCC 17978 and 1656-2. In PCR-1, the upstream and downstream regions of the *ompA* gene in the bacterial strains were amplified with the locus-specific primers shown in Table 2 (Fig. 6B). *ermAM*, conferring erythromycin resistance, was amplified using primer pair U3/U4 (Table 2) with pIL252 as the template. In PCR-2, the approximately 3.1-kb and 3.3-kb DNA fragments comprising the upstream and downstream regions of *ompA* and *ermAM*, respectively, were combined by overlap extension PCR (Fig. 6B); they were then gel purified and cloned into *FspI*-digested pHKD01 to result in pHKD14 (*A. baumannii* ATCC 17978 $\Delta\text{ompA}::\text{ermAM}$) and pHKD15 (*A. baumannii* 1656-2 $\Delta\text{ompA}::\text{ermAM}$) (Table 1). The *ompA* gene was deleted in each strain by using a conjugation-based gene transfer system and homologous recombination. In order to confirm the deletion of the target gene on the chromosomes of *A. baumannii* ATCC 17978 and 1656-2, PCR analysis was performed using primer pairs OMPA101F/OMPA102R and OMPA201F/OMPA202R (Table 2). PCR analysis of the genomic DNA of each wild-type strain indi-

cated fragment sizes of 3.1 kb and 3.4 kb (Fig. 6C). However, the genomic DNAs from the *ompA* mutants, HKD14 and HKD15, yielded amplified DNA fragments of approximately 2.1 kb and 2.3 kb (Fig. 6C). These results indicated that the method proposed in the present study could be applied to *A. baumannii* clinical isolates that present different resistances to antibiotics without the construction of new vectors carrying suitable antibiotic resistance cassettes. Furthermore, the effects of *ompA* mutation on the biofilm-forming abilities of *A. baumannii* strains ATCC 17978 and 1656-2 were assessed using gentian violet staining (Fig. 7). In comparison to biofilm formation by the parental wild-type strains, the abilities of the *ompA* mutant strains to form biofilms were significantly reduced on polystyrene surfaces as visualized by using a digital imaging system (Fig. 7A). In quantitative biofilm analysis, the biofilm-forming abilities (calculated as $\text{OD}_{570}/\text{OD}_{600}$) of the wild-type strains, ATCC 17978 and 1656-2, were 0.8 and 3.6, whereas the abilities of the *ompA* mutant strains, HKD14 and HKD15, were 0.2 and 0.1, respectively (Fig. 7B). These results suggested that *OmpA* is likely to play an important role in biofilm formation in both strains.

DISCUSSION

Unmarked gene deletion by suicide vectors has become an invaluable tool in the study of gene functions in the pathogenesis of *A. baumannii* (12–14), because gene disruption by the insertion of antibiotic resistance markers causes significant difficulties in the complementation of the mutant and the introduction of additional mutations into multidrug-resistant *A. baumannii* strains. However, in the process of DNA cloning, limited choices of restriction sites result in problems in the construction of chimeric plasmids. To eliminate this obstacle, commercially available kits (the Gibson Assembly cloning kit and the GeneArt Seamless assembly kit) have been developed. The kits are convenient because the methods enable cloning of the overlap DNA fragments directly into a vector linearized by restriction digestion, as well as successful assembly of multiple DNA fragments. However, this approach also has drawbacks. First, self-ligation of the digested plasmids

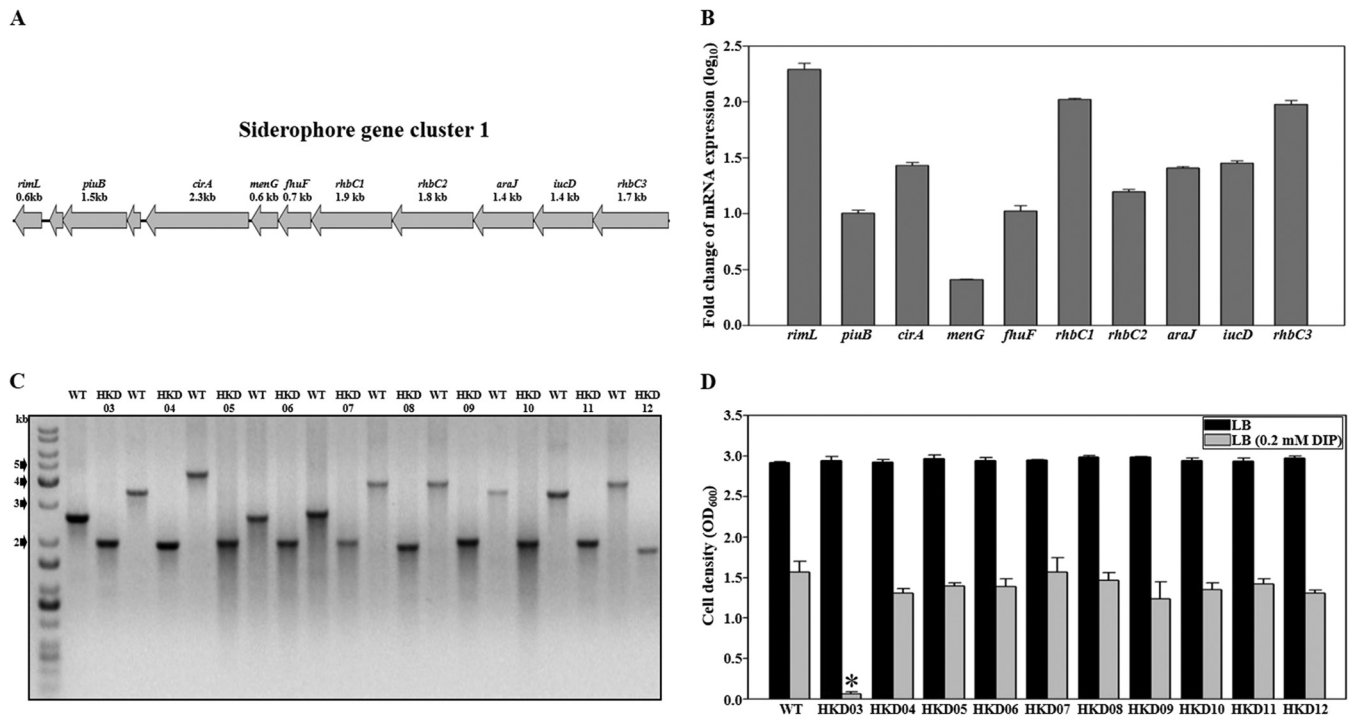


FIG 4 Functional analysis of siderophore gene cluster 1. (A) Siderophore gene cluster 1 (locus tags, HMPREF0010_00723 to -00734), selected as target genes for the construction of deletion mutants. The size of the open reading frame of each gene to be deleted is given. Locus tags are based on the database of the *A. baumannii* ATCC 19606 genome sequence that was retrieved from GenBank (accession number [ACQB000000000](https://www.ncbi.nlm.nih.gov/GenBank/ACQB000000000)). (B) The mRNA levels of *rimL*, *piuB*, *cirA*, *menG*, *fluF*, *rhbC1*, *rhbC2*, *araJ*, *iucD*, and *rhbC3* in *A. baumannii* ATCC 19606 were analyzed by qRT-PCR of the total RNA isolated from bacteria grown on LB medium containing 0.2 mM DIP or on LB medium alone. Each bar represents the mRNA level of a gene expressed in bacteria grown on LB medium containing 0.2 mM DIP relative to that in bacteria grown on LB medium alone. Error bars represent SEM. Means and SEM were calculated from three independent experiments. (C) PCR analysis of the wild type and the mutants generated by allelic exchanges. The PCR products from the mutants are approximately 2.0 kb smaller than those from the wild type. (D) The abilities of *A. baumannii* strains to grow on LB medium without or with 0.2 mM DIP were compared. The growth of the bacterial strains was monitored after 10 h. Error bars represent SEM. Means and SEM were calculated from three independent experiments. The asterisk indicates a significant change ($P < 0.0001$) from the result for the wild type cultured in LB medium with 0.2 mM DIP. WT, wild type; HKD03, *rimL* mutant; HKD04, *piuB* mutant; HKD05, *cirA* mutant; HKD06, *menG* mutant; HKD07, *fluF* mutant; HKD08, *rhbC1* mutant; HKD09, *rhbC2* mutant; HKD10, *araJ* mutant; HKD11, *iucD* mutant; HKD12, *rhbC3* mutant.

results in a high background of false-positive colonies without the desired chimeric plasmid, making the selection of true colonies with the desired chimeric plasmid difficult. Second, the application of the method to markerless gene deletion in multidrug-re-

sistant clinical isolates of *A. baumannii* is extremely restricted, because the construction of new vectors carrying a suitable antibiotic resistance marker is absolutely required for the selection of merodiploid strains.

To avoid these drawbacks, in this study, we have developed a rapid and easy cloning method for markerless gene deletion. This method is based on direct insertion of blunt-end PCR products, in which upstream and downstream regions of the target gene are consecutively fused with an antibiotic resistance cassette via overlap extension PCR, into a suicide vector where the same blunt ends have been created by restriction digestion. Importantly, the design is such that the antibiotic resistance cassette is inserted outside the downstream region of the target gene by overlap extension PCR to eliminate the background of false-positive colonies (without desired chimeric plasmids) and facilitate the selection of true colonies (with the desired chimeric plasmids) in DNA cloning. Moreover, this strategy includes removal of the antibiotic resistance cassette via homologous recombination and eliminates the necessity of constructing new vectors containing a suitable antibiotic resistance cassette for markerless gene deletion in multidrug-resistant clinical isolates of *A. baumannii*. To establish the direct cloning of the blunt-end DNA fragments, a new suicide vector was constructed by cloning the double-stranded oligomer containing

TABLE 4 Efficiency of gene deletion by the proposed method

Target gene	No. of merodiploids	No. (%) of colonies		No. (%) of sucrose-resistant colonies	
		Sucrose sensitive	Sucrose resistant	WT ^a	Deletion mutant
<i>basD</i>	50	0 (0)	50 (100)	30 (60)	20 (40)
<i>bauA</i>	50	2 (4)	48 (96)	35 (73)	13 (27)
<i>rimL</i>	50	1 (2)	49 (98)	11 (22.4)	38 (77.6)
<i>piuB</i>	50	2 (4)	48 (96)	18 (37.5)	30 (62.5)
<i>cirA</i>	50	0 (0)	50 (100)	12 (24)	38 (76)
<i>menG</i>	50	2 (4)	48 (96)	32 (66.7)	16 (33.3)
<i>fluF</i>	50	0 (0)	50 (100)	25 (50)	25 (50)
<i>rhbC1</i>	50	1 (2)	49 (98)	38 (77.6)	11 (22.4)
<i>rhbC2</i>	50	0 (0)	50 (100)	30 (60)	20 (40)
<i>araJ</i>	50	1 (2)	49 (98)	17 (34.7)	32 (65.3)
<i>iucD</i>	50	0 (0)	50 (100)	15 (30)	35 (70)
<i>rhbC3</i>	50	2 (4)	48 (96)	38 (79.2)	10 (20.8)

^a WT, wild type.

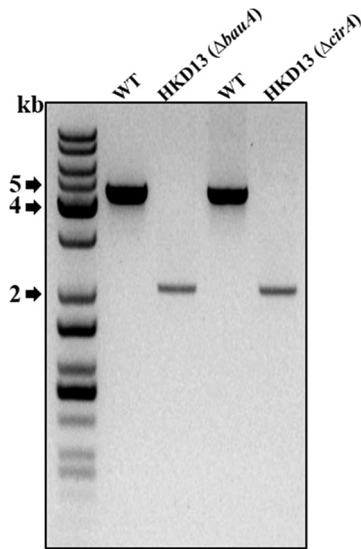


FIG 5 Construction of the double gene deletion mutant. PCR analysis was performed on the genomic DNA from the wild type and double deletion mutant HKD013 with primer pairs BAUA01F/BAUA02R and CIRA01F/CIRA02R. WT, wild type; HKD13, *bauA cirA* double deletion mutant.

the *FspI* blunt-end site into pDS132. For the validation of the proposed method, *basD* and *bauA* were selected as the target genes to be deleted, and markerless deletion of these genes was successfully achieved by using the proposed method.

Iron is one of the major limiting factors for pathogenic bacteria in host cells (23). For iron acquisition, pathogens regulate the expression of genes involved in the production of high-affinity iron-chelating compounds known as siderophores in response to the level of iron availability (24). Genes required for the biosynthesis, efflux, and uptake of siderophores are commonly clustered within bacterial genomes (23). Siderophore gene cluster 1, in

which several genes likely to be involved in the biosynthesis (*RimL*, *MenG*, *FhuF*, *RhbC1*, *RhbC2*, *IucD*, and *RhbC3*), efflux (*AraJ*), and uptake (*CirA* and *PiuB*) of siderophores are clustered, has been found in *A. baumannii* strains (20). The expression of the genes within *A. baumannii* ATCC 17978 siderophore cluster 1 is significantly upregulated by iron-depleted environmental conditions (20). In agreement with this, we found that the expression of the genes within *A. baumannii* ATCC 19606 siderophore cluster 1 was substantially upregulated by iron-depleted conditions. These results indicated the potential importance of these genes in the biosynthesis, efflux, and uptake of *A. baumannii* siderophores under iron-depleted conditions. Therefore, in the present study, the roles of these genes in the survival of *A. baumannii* under iron-depleted conditions were examined through mutant construction by the proposed method and through phenotypic evaluation of the mutants for survival under iron-depleted conditions. We showed that *rimL*, encoding acetyltransferases, is likely to be essential for the survival of *A. baumannii* under iron-depleted conditions. However, further studies, including complementation testing and investigation of the regulatory mechanisms of *rimL* expression, are needed to clarify the role of *rimL* in the survival of *A. baumannii* under iron-depleted conditions. The effectiveness of the proposed method in double gene deletion was also demonstrated by the additional deletion of *cirA* in a *bauA* mutant.

Furthermore, the usefulness of the proposed method was proved by deletion of the *ompA* gene in *A. baumannii* strains ATCC 17978 and 1656-2. In particular, *A. baumannii* 1656-2 is a clinically important isolate, because its biofilm formation ability and resistance to various antibiotics are remarkable (18, 25). However, the genes involved in these phenotypes have not been characterized, due to the insufficiency of methods for the construction of *A. baumannii* mutants. Thus, the proposed method would help us gain insights into the roles of the genes associated with biofilm formation and antibiotic resistance in multidrug-resistant *A. baumannii* strains.

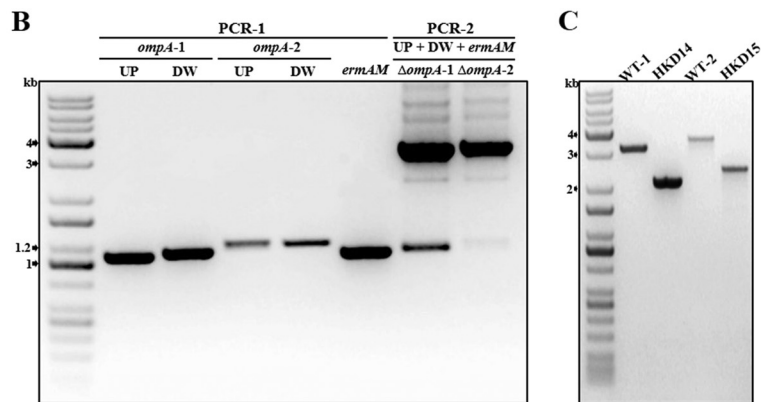
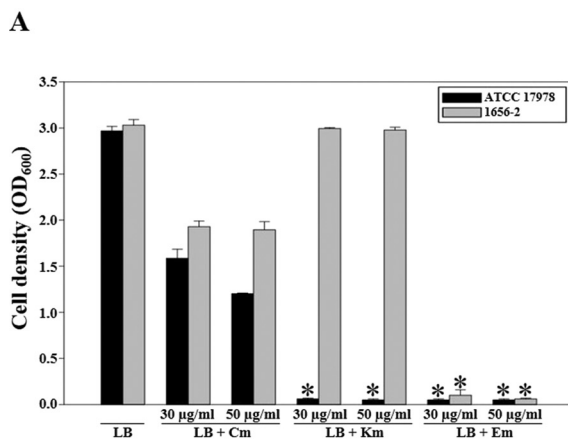


FIG 6 Gene deletion in *A. baumannii* strains other than ATCC 19606. (A) The susceptibilities of *A. baumannii* strains ATCC 17978 and 1656-2 to increasing amounts of antibiotics were explored by measuring their growth after 12 h on LB medium containing different concentrations of antibiotics. Asterisks indicate significant differences ($P < 0.0001$) from the wild-type strains cultured in LB medium. Cm, chloramphenicol; Km, kanamycin; Em, erythromycin. (B) Construction of *ompA* mutants. In PCR-1, the downstream and upstream regions of *ompA* were amplified by PCR using the genomic DNA of *A. baumannii* strain ATCC 17978 or 1656-2. *ermAM*, conferring erythromycin resistance, was amplified by PCR using pIL252 as the template. In PCR-2, each mutated DNA fragment was combined with *ermAM* by overlap extension PCR. *ompA*-1, ATCC 17978 *ompA*; *ompA*-2, 1656-2 *ompA*; UP, upstream region of the target gene to be deleted; DW, downstream region of the target gene to be deleted. (C) PCR analysis of each wild-type and mutant strain generated by allelic exchange. Molecular size markers (1-kb DNA ladder; Kapa) are indicated. WT-1, wild-type ATCC 17978; HKD14, ATCC 17978 *ompA* mutant; WT-2, wild-type 1656-2; HKD15, 1656-2 *ompA* mutant.

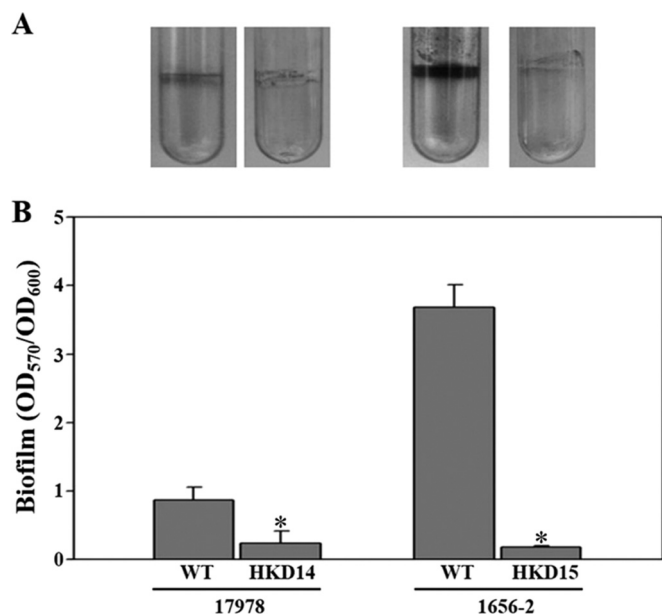


FIG 7 Effects of *ompA* mutation on biofilm formation by *A. baumannii* strains. (A) Biofilms formed on 5-ml polystyrene tubes were stained with gentian violet for 15 min. The remaining biofilms were photographed using a digital imaging system. (B) The amount of gentian violet eluted from the biofilms was quantitated as the OD₅₇₀ normalized to total bacterial growth (OD₆₀₀). Error bars represent SEM. Means and SEM were calculated from three independent experiments. Asterisks indicate significant differences ($P < 0.005$) from the wild-type strains. WT, wild type; HKD14, ATCC 17978 *ompA* mutant; HKD15, 1656-2 *ompA* mutant.

Taking into account the results presented here, the proposed method has several advantages. First, the availability of the restriction site is no longer limited to the cloning steps. Second, recombinant bacteria carrying a desired chimeric plasmid are readily selected on the medium containing the antibiotic corresponding to the antibiotic resistance cassette that is inserted outside the downstream region of the target gene by overlap extension PCR. Third, the antibiotic resistance cassette is easily excised by a second single-crossover homologous-recombination event without any scars. Finally, the method could be used for the introduction of gene deletion in multidrug-resistant clinical isolates of *A. baumannii* without the construction of new vectors containing a suitable antibiotic resistance marker, as well as for multiple gene deletion. Therefore, the proposed method is a useful tool for functional studies of the genes involved in the pathogenesis of multidrug-resistant *A. baumannii*.

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