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In-Frame and Unmarked Gene Deletions in *Burkholderia cenocepacia* via an Allelic Exchange System Compatible with Gateway Technology

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***Burkholderia cenocepacia* is an emerging opportunistic pathogen causing life-threatening infections in immunocompromised individuals and in patients with cystic fibrosis, which are often difficult, if not impossible, to treat. Understanding the genetic basis of virulence in this emerging pathogen is important for the development of novel treatment regimes. Generation of deletion mutations in genes predicted to encode virulence determinants is fundamental to investigating the mechanisms of pathogenesis. However, there is a lack of appropriate selectable and counterselectable markers for use in *B. cenocepacia*, making its genetic manipulation problematic. Here we describe a Gateway-compatible allelic exchange system based on the counterselectable *pheS* gene and the I-SceI homing endonuclease. This system provides efficiency in cloning homology regions of target genes and allows the generation of precise and unmarked gene deletions in *B. cenocepacia*. As a proof of concept, we demonstrate its utility by deleting the *Bcam1349* gene, encoding a cyclic di-GMP (c-di-GMP)-responsive regulator protein important for biofilm formation.**

Burkholderia cenocepacia is a member of a group of closely related Gram-negative bacteria referred to as the *Burkholderia cepacia* complex (Bcc). The Bcc contains at least 18 different species that thrive in diverse ecological niches, including clinical, industrial, and natural environments. These bacteria possess very large genomes separated into multiple replicons and hence are considered one of the most versatile groups of Gram-negative bacteria (1, 2). Some Bcc species have biotechnological potential for use in processes such as the enhancement of plant growth or breakdown of pollutants, while others are opportunistic pathogens causing life-threatening infections in immunocompromised individuals and in patients with cystic fibrosis (CF) (3). Although all members of the Bcc have been isolated from CF patients, *B. cenocepacia* accounts for the majority of these isolates, comprising the most virulent and transmissible strains associated with a poor clinical course and a high mortality rate (4). Therefore, research on the virulence mechanisms of Bcc bacteria has focused largely on *B. cenocepacia*.

The genomes of several *B. cenocepacia* strains have recently been sequenced (5–7), enabling bioinformatics-based predictions of virulence determinants in this pathogen. Although a number of genes associated with virulence in *B. cenocepacia* have been identified (4, 8, 9) and tested in various infection models (10, 11), it seems likely that the list of genes implicated in virulence is far from complete and will expand with genetic tools becoming available to manipulate *B. cenocepacia* strains. The deletion of genes potentially associated with virulence is a powerful way to investigate their function in bacterial physiology and pathogenesis. Most of the virulence traits of *B. cenocepacia*, such as antibiotic resistance, motility, biofilm formation, cell invasion, and intracellular survival, are multifactorial, involving more than one gene; thus, multiple gene deletions may need to be generated in one strain to fully assess the genetic basis of a particular virulence trait. This requires an efficient method to generate gene deletions, which are preferably not marked with antibiotic resistance cassettes, as this would prevent the ability to mutate more than a single gene in one particular strain and moreover may cause polar effects on adjacent genes. During the past few years, a number of elegant systems have been developed for the generation of unmarked gene deletions in *B. cenocepacia* (12, 13) as well as in other *Burkholderia* species (14–16). In these systems, regions of homology containing a mutant allele of a target gene are cloned into a suicide vector. These vectors are then transferred into the bacterial host by conjugation. The integration of the plasmid into the chromosome by homologous recombination is selected by antibiotic resistance encoded by a gene on the plasmid, leading to the formation of merodiploids, which contain both the mutant and wild-type alleles of the target gene. The resolution of merodiploids by the excision of the integrated plasmid in a second homologous recombination event results in a population of cells of which a significant fraction contains the desired gene deletion. The latter step usually requires counterselection for the integrated plasmid since the second homologous recombination can be an exceptionally rare event.

Sucrose counterselection based on the *sacB* gene (15, 17) and an engineered counterselectable marker based on the *Burkholderia pseudomallei pheS* gene encoding the α-subunit of phenylalanyl tRNA synthase (14) have been used in some *Burkholderia*

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Laboratory identification no.	Relevant characteristic(s)	Reference or source
Strains			
B. cenocepacia H111	MF108	Clinical isolate from a cystic fibrosis patient	7
B. thailandensis CDC2721121		Clinical isolate from a patient with pleural infection	27
E. coli DH5α	TTN322	Used for standard DNA manipulations	Invitrogen
E. coli DB3.1	TTN312	Host for the Gateway-compatible gene replacement vectors	Invitrogen
Plasmids			
pBBR1MCS5	MF528	Broad-host-range cloning vector; Gm^r	22
pBBR1MCS2	MF124	Broad-host-range cloning vector; Km^r	22
pMF564	MF564	*Bcam1349* gene cloned into pBBR1MCS5	This study
pYedQ	MF202	*E. coli yedQ* (*yhcK*) gene cloned into pRK404A	21
pYedQ2	MF217	*yedQ* gene cloned into the HindIII/BamHI site in pBBR1MCS5	This study
pRK600	TTN365	Helper plasmid in triparental conjugations; Cm^r *ori*-ColE1 RK-*mob*$^+$ RK-*tra*$^+$	28
pDONR221	TTN313	Source of Gateway donor site, Gateway donor vector; Km^r	Invitrogen
pBBR1MCS-Km-*pheS*	MF138	Engineered *pheS* cloned into pBBR1MCS2; Km^r	14
pEX18Tp-*pheS*	MF322	Gene replacement vector based on *pheS* and Tp^r	14
pEX18Gm-*pheS*	MF320	Gene replacement vector based on *pheS* and Gm^r	14
pEX18Km-*pheS*	MF321	Gene replacement vector based on *pheS* and Km^r	14
pUC57-*pheS*	MF130	Cloning vector containing engineered *pheS*; Ap^r	14
pDAI-SceI	MF339	Cloning vector containing the I-SceI endonuclease; Tet^r	12
pDONRPEX18Tp-SceI-*pheS*	MF415	~2.6-kb Gateway donor site cloned into the XbaI/HindIII site of pEX18Tp-*pheS*; Tp^r	This study
pDONRPEX18Gm-SceI-*pheS*	MF356	~2.6-kb Gateway donor site cloned into the XbaI/HindIII site of pEX18Gm-*pheS*; Gm^r	This study
pDONRPEX18Km-SceI-*pheS*	MF414	~2.6-kb Gateway donor site cloned into the XbaI/HindIII site of pEX18Km-*pheS*; Km^r	This study
pENTRPEX18Tp-SceI-*pheS*-*Bcam1349*	MF455	Gene replacement vector containing the *Bcam1349* deletion allele; Tp^r	This study
pENTRPEX18Tp-SceI-*pheS*-*phzF*	MF450	Gene replacement vector containing the *phzF* deletion allele; Tp^r	This study
pDAI-SceI-*pheS*	MF355	~1.2-kb XbaI/SphI *pheS* fragment from pUC57-*pheS* cloned into the XbaI/SphI site of pDAI-SceI	This study

species. However, they appear to be inappropriate and leaky counterselectable markers for the generation of *B. cenocepacia* gene deletions in our laboratory. Another way to stimulate the second homologous recombination event and, consequently, the resolution of merodiploids is based on the yeast homing endonuclease I-SceI, which recognizes a specific 18-bp sequence (12, 15). After an allelic exchange vector carrying the I-SceI recognition site has integrated into the chromosome, a replicative second plasmid constitutively expressing the I-SceI enzyme is introduced into the merodiploid bacteria. The I-SceI enzyme creates a double-stranded DNA break at the I-SceI site within the integrated plasmid, which stimulates a second homologous recombination event by the host's DNA repair system. The excision of the integrated plasmid results in a population of cells carrying either the wild-type or the mutant allele, which can be identified by PCR and partial sequencing.

Another major limitation of allelic exchange vectors for *Burkholderia* species is their dependence on restriction and ligation enzymes for cloning. Restriction-free cloning based on Gateway recombineering technology (18) is an alternative method that can expedite the construction of gene replacement vectors containing mutant alleles.

Here we present a Gateway-compatible allelic exchange system for *Burkholderia* species that utilizes the I-SceI homing endonuclease and *pheS*-based counterselection. We further describe the application of this system for generating in-frame and unmarked gene deletions in *B. cenocepacia* H111. As a proof of concept, we describe the deletion and complementation of the *Bcam1349* gene, which is a regulator of biofilm formation in *B. cenocepacia* H111. In addition, we also provide evidence that this system can be used to make gene deletions in *Burkholderia thailandensis*, indicating that it may be used in other *Burkholderia* species as well.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All *B. cenocepacia* and *Escherichia coli* strains were grown at 37°C. Luria broth (LB) medium was used for overnight batch cultivation of all bacteria unless otherwise stated. Solid media were prepared with 2% (wt/vol) agar. Eighty micrograms tetracycline (Tet) ml^{-1} (liquid medium), 120 μg μg Tet ml^{-1} (solid medium), 25 μg gentamicin sulfate (Gm) ml^{-1}, 100 μg kanamycin sulfate (Km) ml^{-1}, and 100 μg trimethoprim (Tp) ml^{-1} were used for *B. cenocepacia* strains, and 20 μg Tet ml^{-1}, 10 μg Gm ml^{-1}, 50 μg Km ml^{-1}, 50 μg Tp ml^{-1}, 100 μg ampicillin (Ap) ml^{-1}, and 25 μg chloramphenicol (Cm) ml^{-1} were used for *E. coli* strains where appropriate. After conjugal transfer of plasmids into *B. cenocepacia*, AB agar medium (19) supplemented with 10 mmol $liter^{-1}$ Na-citrate and appropriate antibiotics was used to select for *B. cenocepacia* transconjugants. For self-curing of plasmid pDAI-SceI-*pheS*, 0.1% (wt/vol) *p*-chlorophenylalanine (cPhe) (DL-4-chlorophenylalanine; Sigma-Aldrich) was autoclaved together with B-salts solution and A-salts solution (19), and the carbon source of choice was added thereafter.

Construction of Gateway-compatible allelic exchange vectors. The *attB1*- and *attB2*-flanked Gateway donor site was amplified by PCR from pDONR221 by using primers GWE-SceI-F (flanked by HindIII and I-SceI restriction sites) and GWE-R (flanked by the XbaI site). The resulting 2.6-kb PCR product was digested with HindIII and XbaI and cloned into HindIII/XbaI-digested plasmids pEX18Tp-*pheS*, pEX18Gm-*pheS*, and pEX18Km-*pheS* (14), resulting in the allelic exchange vectors pDONRPEX18Tp-SceI-*pheS*, pDONRPEX18Gm-SceI-*pheS*, and pDONRPEX18Km-SceI-*pheS*, respectively (Fig. 1). The insertion of the Gateway donor site was confirmed by restriction analysis and partial sequencing of the newly generated vectors. These vectors are maintained in *E. coli* strain DB3.1, which contains a *gyrA462* mutation (Invitrogen).

Construction of the I-SceI expression vector pDAI-SceI-*pheS*. To construct pDAI-SceI-*pheS* (Fig. 1), an ~1.2-kb fragment containing the *pheS* gene was excised from pUC57-pheS (14) by restriction with XbaI and

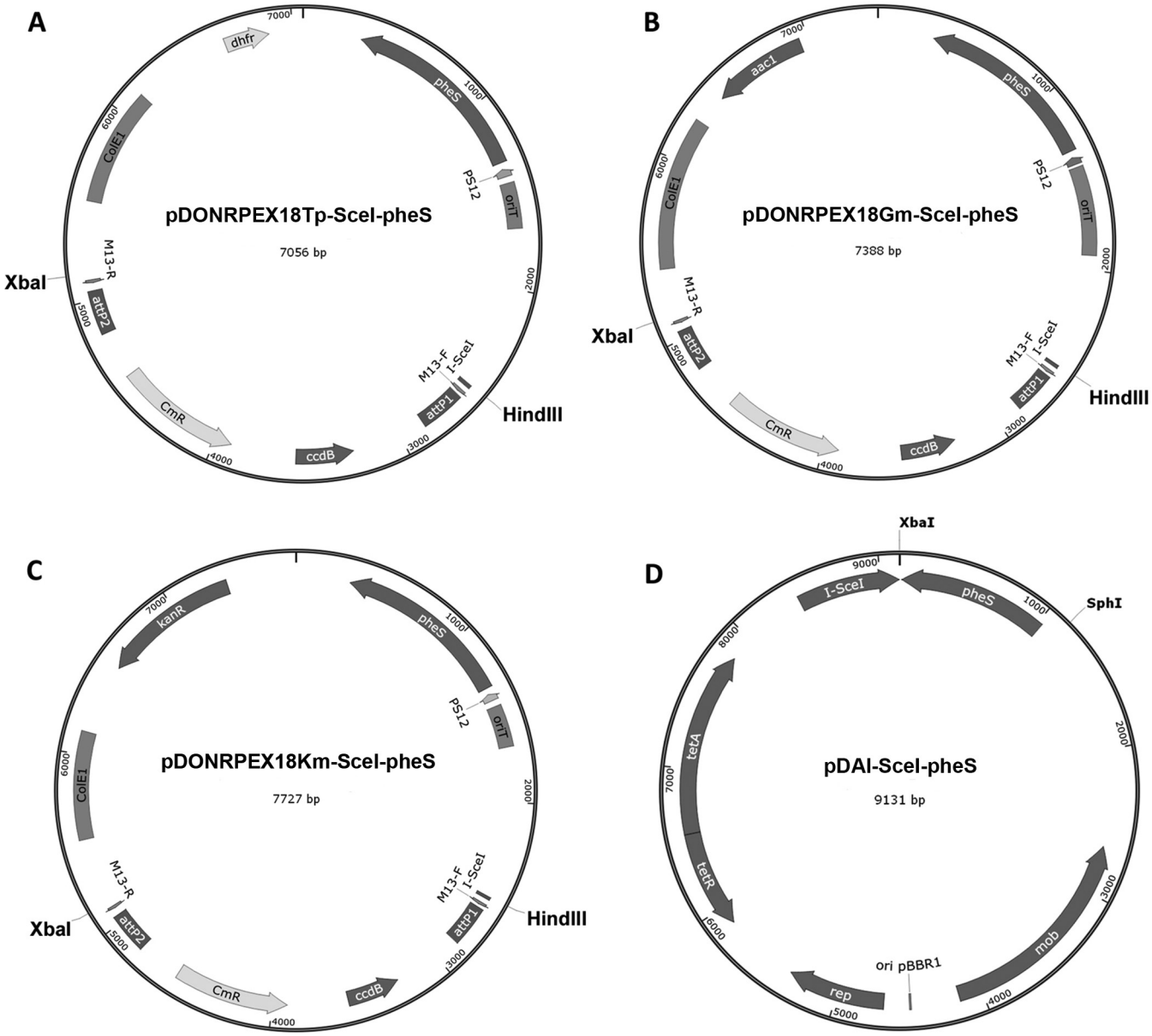

FIG 1 Maps of the allelic exchange vectors and the I-SceI expression vector constructed in this study. (A to C) Gene replacement vectors, each containing a different antibiotic resistance marker, were constructed by cloning the Gateway donor site into the XbaI/HindIII site of a set of pEX family vectors based on the mutant *pheS* gene (14). *attP1* and *attP2*, lambda recombination sites; CmR, chloramphenicol acetyltransferase; *ccdB*, gene encoding a gyrase-modifying enzyme; *dhfr*, dihydrofolate reductase-encoding gene; *aac1*, Gm acetyltransferase-encoding gene; *kanR*, gene conferring resistance to kanamycin; *pheS*, mutant gene for the α-subunit of phenylalanyl tRNA synthase; P_{S12}, *B. pseudomallei rpsL* gene promoter; I-SceI, I-SceI endonuclease recognition site; ColE1, origin of replication; *oriT*, conjugal origin of transfer; M13-F and M13-R, primer binding sites for partial sequencing of the DNA sequence cloned into *attP1-attP2* sites. (D) pDAI-SceI-*pheS* was constructed by cloning the *pheS* gene into the XbaI/SphI site of plasmid pDAI-SceI (12). *tetA* and *tetR*, genes encoding the tetracycline-specific efflux protein and repressor protein, respectively; *mob*, region facilitating conjugal transfer; I-SceI, I-SceI endonuclease; *ori* pBBR1, origin of replication; *rep*, gene encoding the pBBR1 replication protein.

SphI and was ligated into XbaI/SphI-digested plasmid pDAI-SceI (12). The presence of the insertion was verified by restriction analysis.

Construction of the gene replacement vector pENTRPEX18Tp-SceI-*pheS-Bcam1349*. The upstream fragment of the *Bcam1349* gene was amplified by using primers Bcam1349-UpF-GWR and Bcam1349-UpR-tail, and the downstream fragment of the *Bcam1349* gene was amplified by using primers Bcam1349-DnF and Bcam1349-DnR-GWL (Table 2). Both fragments were amplified by using Phusion high-fidelity DNA polymerase (Thermo Scientific) according to the manufacturer's instructions and under the following thermal cycling conditions: 98°C for 2 min; 25 cycles of 98°C for 15 s, 64°C for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 7 min. The PCR fragments were purified by using the Wizard SV gel and PCR Clean-Up system (Promega), and their concentrations were determined spectrophotometrically. The up- and downstream fragments were fused together and amplified by using primers GW-*attB*1 and GW-*attB*2 (Table 2) in splicing by overlap extension PCR (SOE PCR) (20) to generate the *Bcam1349* mutant allele as follows. Equal amounts (50 ng) of each up- and downstream fragment and the other components of the PCR mixture except

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)
Gene specific	
Bcam1349-UpF-GWL[a]	TACAAAAAAGCAGGCTAACGGGGATTTCGCACGAT
Bcam1349-UpR-tail[b]	**GGACATCGACTGCATCGTCA**AGCTCGAGTGAAGATGAAGCA
Bcam1349-DnF	TGACGATGCAGTCGATGTCC
Bcam1349-DnR-GWR[a]	TACAAGAAAGCTGGGTGAGATTGATCGCCGGCAT
Common[c]	
GW-*attB*1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
GW-*attB*2	GGGGACCACTTTGTACAAGAAAGCTGGGT
Amplification of the Gateway donor site	
GWE-SceI-F[d]	TACTACAAGCTT**TAGGGATAACAGGGTAAT**AGCATGGATGTTTTCCCAGT
GWE-R[d]	TACTACTCTAGATCAGAGATTTTGAGACACGGG
Other[e]	
Bcam1349-F	TACTACCCCGGGTAAATCGCTTATTCGGGCTG
Bcam1349-R	TACTACTCTAGACATTCGTTCCACCGGACAT
Bcam1349-RBS-F	TACTACTCTAGAATTGTCCGGAAATGGATTGGT
Bcam1349-RBS-R	TACTACCCCGGGATTCGTTCCACCGGACAT

[a] Double-underlined sequences are common for all genes amplified and overlap the GW-*attB* primer sequences (29).
[b] The sequence in boldface type overlaps the gene-specific DnF primer.
[c] Sequences were obtained from reference 29.
[d] Restriction enzyme sites are single underlined, and the I-SceI endonuclease recognition site is in boldface type and double underlined.
[e] Restriction enzyme sites are underlined.

primers GW-*attB*1 and GW-*attB*2 were mixed. PCR was carried out under the following thermal cycling conditions: 98°C for 2 min; 3 cycles of 98°C for 15 s, 64°C for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 1 min. The final extension step was paused at 30 s, primers GW-*attB*1 and GW-*attB*2 were added, and thermal cycling was continued, with 27 cycles of 98°C for 15 s, 64°C for 30 s, and 72°C for 2 min and a final extension step of 72°C for 7 min. The PCR product was then purified and verified by restriction analysis.

A BP clonase reaction for the recombinational transfer of the mutant allele into the allelic exchange vector pDONRPEX18Tp-SceI-*pheS* was performed at 25°C overnight according to the Gateway cloning manual (Invitrogen), using only one-half the recommended amount of BP clonase II enzyme mix (Invitrogen). The BP clonase reaction product was transferred into chemically competent *E. coli* DH5α cells. The transformants growing on LB agar plates containing 50 μg Tp ml^{-1} were screened by colony PCR using primers GWE-SceI-F and GWE-R for insertion of the deletion allele. A number of positive clones were streaked onto LB agar plates containing 50 μg Tp ml^{-1} for purification, plasmid isolation, and partial sequencing.

Construction of pYedQ2 and complementation plasmid pMF564. Plasmid pYedQ2, which was used to elevate intracellular cyclic di-GMP (c-di-GMP) levels, was constructed as follows. The *yedQ* expression cassette was excised from plasmid pYedQ (21) by restriction with BamHI and HindIII and was inserted into the BamHI/HindIII-digested broad-host-range cloning vector pBBR1MCS-5 (22). The presence of the insertion was confirmed by restriction analysis.

Complementation plasmid pMF564 was constructed as follows. The vector pBBR1MCS-5 was digested with SphI and blunt ended by T4 DNA polymerase. The linearized vector was further digested with XbaI and dephosphorylated with shrimp alkaline phosphatase. The SphI/XbaI digestion removed the P_{lac} promoter and the related regulatory sequences from the plasmid. An ~1.5-kb fragment containing the *Bcam1349* gene and its ~0.7-kb upstream DNA sequence was PCR amplified by using primers Bcam1349-RBS-F and Bcam1349-RBS-R, which were flanked by SmaI and XbaI restriction sites, respectively. The PCR fragment was digested with SmaI and XbaI and cloned into the previously linearized vector, yielding complementation plasmid pMF564. The presence of the insertion was confirmed by restriction analysis.

Mutagenesis of *B. cenocepacia* H111. The gene replacement vector pENTRPEXTp-SceI-*pheS*-*Bcam1349* was introduced by conjugation into *B. cenocepacia* via triparental mating, as described previously (23). The cointegrants were selected for Tp resistance on AB-citrate agar plates containing 100 μg Tp ml^{-1}. Four Tp-resistant colonies were streaked onto the same selective plates, and the growing colonies were screened for the integration of the plasmid by colony PCR using primers Bcam1349-F and Bcam1349-R (Table 2). A single positive merodiploid clone was transformed with pDAI-SceI-*pheS* by triparental mating to stimulate the second homologous recombination event and resolve the merodiploid state. The transconjugants were screened for Tet resistance on AB-citrate agar plates containing 120 μg Tet ml^{-1}. Batches of 10 Tet-resistant colonies were screened for the loss of the wild-type allele and the presence of the desired gene deletion by colony PCR using primers Bcam1349-F and Bcam1349-R. Two positive clones were purified by streaking and growing the clones on an AB-citrate agar plate. Thereafter, a single colony for each clone was picked and grown in 1 ml AB-glucose medium containing 0.1% (wt/vol) cPhe at 37°C overnight in order to stimulate the loss of pDAI-SceI-*pheS* via the counterselectable marker *pheS* on the plasmid. Tenfold serial dilutions of the cultures grown overnight were plated onto LB agar plates without any antibiotic, and 20 of the growing colonies of each clone were patched onto LB agar plates with or without tetracycline by using a sterile toothpick to screen for Tet sensitivity, which indicated the loss of plasmid pDAI-SceI-*pheS*. A single positive colony for each clone was selected and stored at −80°C.

Phenotypic characterization of the *B. cenocepacia Bcam1349* deletion mutant. Colony morphology, pellicle formation, and flow cell biofilm formation assays were performed as described previously (23).

RESULTS AND DISCUSSION

Features of the Gateway-compatible allelic exchange vectors. The allelic exchange vectors pEX18Tp-*pheS*, pEX18Gm-*pheS*, and pEX18Km-*pheS*, which contain different antibiotic resistance markers, were first described by Barrett and colleagues (14). These vectors are derivatives of a set of pEX family vectors (25), in which the counterselectable marker gene *sacB* was replaced with a mutant allele of the *B. pseudomallei pheS* gene. Here we modified

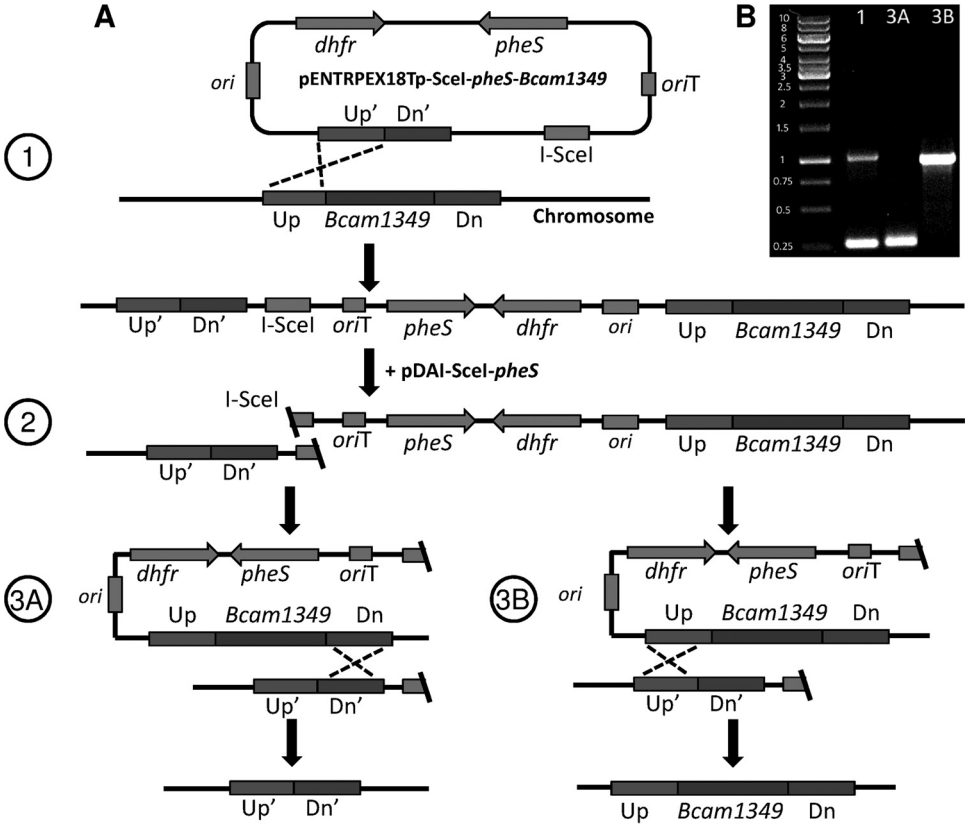

FIG 2 Schematic diagram depicting the gene replacement procedure in *B. cenocepacia* H111 (A) and gel image (B). In step 1, the gene replacement vector pENTRPEX18Tp-SceI-*pheS*-*Bcam1349* (derivative of pDONRPEX18Tp-SceI-*pheS*) contains regions of homology flanking the *Bcam1349* gene. The vector was transferred into *B. cenocepacia* by conjugation and integrated into the chromosome by the first homologous recombination event, resulting in trimethoprim-resistant merodiploids, which were verified by colony PCR (B, lane 1). In step 2, the merodiploid was transformed with pDAI-SceI-*pheS*. The I-SceI endonuclease expressed from the plasmid introduces a double-stranded DNA break at the I-SceI recognition site on the chromosome. In step 3, The DNA break stimulates the second homologous recombination event through the host DNA repair system. Depending on the location of the second recombination event, the resolution of the merodiploid state either generates the desired gene deletion (step 3A [A] and lane 3A [B]) or restores the wild-type allele (step 3B [A] and lane 3B [B]), which is identified by colony PCR.

these vectors for use as Gateway-compatible donor vectors to clone regions of homology containing the deleted allele of a target gene. This was carried out by cloning the Gateway donor site from pDONR221 into the multicloning site of the above-mentioned vectors. The 18-bp I-SceI recognition site was incorporated into the vectors as a tail to the forward primer during PCR amplification of the donor cassette. The resulting vectors (Fig. 1) contain *attP1* and *attP2* sequences required for recombination-based cloning and the *ccdB* gene as a counterselectable marker, which kills *gyrA*$^+$ host cells, such as *E. coli* DH5α cells, by inducing gyrase-mediated double-stranded DNA breaks, providing positive selection for *E. coli* clones bearing plasmids with cloned inserts. Additionally, the vectors contain the counterselectable *pheS* gene (14) driven by the P_{S12} promoter of the *B. pseudomallei rpsL* gene (26) and the I-SceI recognition site for downstream resolution of merodiploids. Although the mutant *pheS* gene was shown to be efficient in killing *Burkholderia thailandensis* cells in the presence of cPhe when expressed as a single copy from the gene replacement vector integrated on the chromosome (14), it was inefficient in killing *B. cenocepacia* H111 cells, and the resolution of merodiploids was almost impossible when the cells were grown in the presence of cPhe. Therefore, we incorporated the I-SceI site into the gene replacement vectors for downstream resolution of merodiploids. We preferred to keep the *pheS* gene on the gene replacement vectors, as it can efficiently be utilized as a counterselectable marker in strains such as *B. thailandensis* (14).

Features of the I-SceI expression vector pDAI-SceI-*pheS*. The vector pDAI-SceI-*pheS* (Fig. 1), which constitutively expresses the I-SceI endonuclease, is a derivative of the vector pDAI-SceI, the features of which were previously described by Flannagan and colleagues (12). Although the mutant *pheS* gene was not efficient in killing *B. cenocepacia* cells in the presence of cPhe when expressed as a single copy on the chromosome, it effectively killed almost all *B. cenocepacia* cells when expressed from the multicopy plasmid pBBR1MCS-Km-*pheS* (14) (see Fig. S1 in the supplemental material), indicating that the mutant *pheS* gene has to be present in multiple copies in the cells to provide effective counterselection in *B. cenocepacia*. Based on this finding, we modified pDAI-SceI by cloning the mutant *B. pseudomallei pheS* gene from pUC57-*pheS* (14) into the multicloning site of pDAI-SceI to expedite self-curing of the plasmid. In the presence of 0.1% cPhe, the mutant *pheS* gene enables efficient killing of *B. cenocepacia* cells containing pDAI-SceI-*pheS* and curing of the *B. cenocepacia* deletion mutants from the plasmid once they are obtained after the resolution of merodiploids. In this way, the deletion mutants become ready for subsequent rounds of mutagenesis.

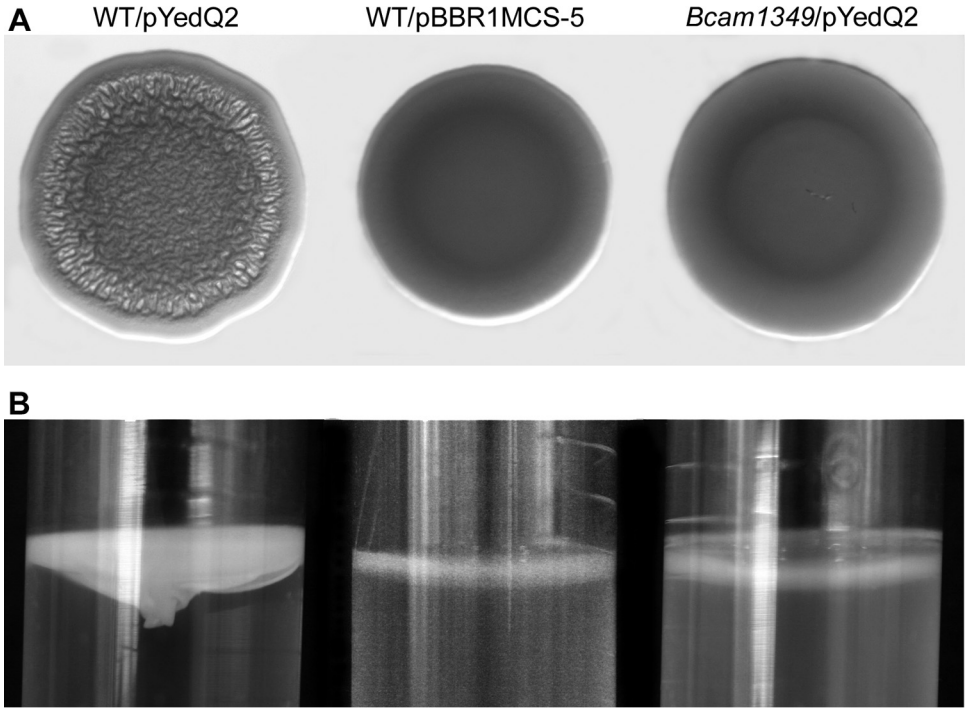

FIG 3 Phenotypic characterization of the *Bcam1349* deletion mutant. Shown are colony morphology on AB-glucose agar medium (A) and pellicle formation in static LB liquid culture (B) of the wild-type (WT) and *Bcam1349* mutant strains carrying pYedQ2 and the wild-type strain carrying pBBR1MCS-5 (vector control).

Construction of the *B. cenocepacia Bcam1349* deletion mutant. Using the allelic exchange system described here, we have successfully generated gene deletions in both *B. cenocepacia* H111 and *B. thailandensis* (see the supplemental material). As a proof of concept, we present a procedure that was used to delete the *Bcam1349* gene. This gene encodes a c-di-GMP-responsive CRP/FNR superfamily transcription factor and regulates biofilm formation in *B. cenocepacia* H111 (23, 24). We previously showed that elevated intracellular levels of c-di-GMP promoted wrinkled-colony formation on solid medium, robust pellicle formation at the air-liquid interface of static liquid cultures, and increased biofilm formation in flow cells. However, despite having high intracellular c-di-GMP levels, a transposon insertion mutant of *Bcam1349* did not form wrinkled colonies, pellicles, or thick flow cell biofilms (23).

We created the *Bcam1349* mutant allele in two consecutive PCR rounds using three primer pairs (Table 2). Two of these primer pairs were gene specific, and one of them was common and can be used routinely. Gene-specific primers were designed to amplify fragments ranging from 0.8 to 1 kb in size. The fragments were chosen so that the gene-specific UpF-GWR primer is placed within 10 to 100 bp after the gene start and the gene-specific primer DnR-GWL is placed within 10 to 100 bp before the stop codon. The gene-specific primers were compared to the *B. cenocepacia* H111 genome to make sure that they would not fully anneal to unspecific regions in the genome. In the first PCR round, the gene-specific primers were used to amplify up- and downstream homology regions of the target gene. We usually obtained single major PCR products of the correct size, which were subsequently purified with a PCR cleanup kit and used in the second PCR round. However, if there are multiple bands, the entire PCR mixtures should be loaded onto an agarose gel, and fragments with the correct size should be gel extracted. In the second PCR round, equal amounts of up- and downstream PCR fragments were fused together and amplified with the common primers GW-*attB*1 and GW-*attB*2 (Table 2), incorporating the *attB1* and *attB2* recombination sites at either end of the deletion allele. We usually obtained a single major PCR product of the correct size (~2 kb) at this step.

We recombined the *Bcam1349* mutant allele into pDONRP EX18Tp-SceI-pheS using BP clonase and transferred the entire BP reaction product into *E. coli* DH5α cells. Tp-resistant transformants were selected, and the presence of the correct plasmid was checked by colony PCR using primers GWE-SceI-F and GWE-R. Alternatively, M13-F and M13-R primers can be used. Plasmids were isolated from a number of positive clones, and the presence of the deletion allele was verified by restriction analysis and partial sequencing.

The resulting gene replacement vector, pENTRPEX18Tp-SceI-*pheS-Bcam1349*, was transferred into *B. cenocepacia* by triparental mating, giving rise to Tp-resistant merodiploids (Fig. 2A). The integration of the nonreplicative vector into the chromosome can normally be verified by colony PCR using gene-specific primers UpF-GWR and DnR-GWL, often resulting in two PCR products corresponding to the wild-type and deletion alleles (Fig. 2B). However, we had to use another pair of primers, Bcam1349-F and Bcam1349-R, to verify the integration of the vector, as the former primer pair did not result in any PCR products. During the generation of deletion mutants of other genes, we also noticed that it is not always possible to see a PCR product corresponding to the wild-type allele, as its amplification may not be favored due to its relatively large size compared to that of the deletion allele. A single

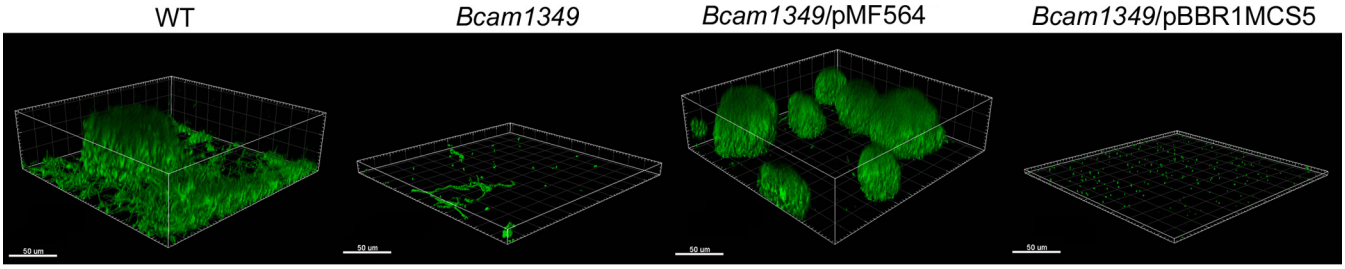

FIG 4 Flow cell biofilm formation by the wild type (WT), the *Bcam1349* mutant and its complemented counterpart, and vector control strains. Confocal laser scanning microscopy images were acquired after 24 h of incubation at 37°C.

merodiploid clone was selected and transformed with pDAI-SceI-*pheS* by conjugation to stimulate the second homologous recombination event via the generation of a double-stranded DNA break by I-SceI endonuclease expressed from the plasmid. Depending on the location of the second recombination event, the resolution of the merodiploid state either restored the wild-type allele or generated the desired gene deletion (Fig. 2A). Eight Tet-resistant colonies were selected and verified for *Bcam1349* deletion by colony PCR. In our experience, at least one colony always contained the desired gene deletion (Fig. 2B). Finally, the deletion mutant was cured from plasmid pDAI-SceI-*pheS* by growing the mutant in liquid medium containing 0.1% cPhe, as described in Materials and Methods. The counterselection medium with cPhe should not contain any competing phenylalanine for efficient counterselection. We therefore prefer to use AB minimal medium supplemented with glucose as a carbon source. However, in the case of deleting genes essential for growth in minimal medium, the mutants can alternatively be cured from plasmid pDAI-SceI-pheS by growing them in serial passages in rich medium without cPhe and Tet, which is required for the maintenance of the plasmid.

Phenotypic characterization of the *Bcam1349* deletion mutant. We previously demonstrated that a transposon insertion mutant of *Bcam1349* did not form wrinkled colonies, robust pellicles, or thick flow cell biofilms despite having high intracellular c-di-GMP levels (23). To characterize the *Bcam1349* deletion mutant obtained here, we first transformed it with plasmid pYedQ2, which contains the *E. coli* diguanylate cyclase protein YedQ and leads to elevated intracellular levels of c-di-GMP in *B. cenocepacia* (23). Unlike the pYedQ2-containing wild type, the pYedQ2-containing *Bcam1349* mutant formed smooth colonies on AB agar medium (Fig. 3A) and did not form robust pellicles in static liquid culture (Fig. 3B). Furthermore, we tested the biofilm formation ability of the *Bcam1349* mutant in a flow cell biofilm system. In accordance with the above-described results, the *Bcam1349* mutant was markedly impaired in biofilm formation compared to the wild-type strain (Fig. 4). To rule out the possibility that the observed biofilm defect was due to a secondary mutation obtained during the mutagenesis procedure, we genetically complemented the mutant strain with an intact copy of the *Bcam1349* gene and its 0.7-kb upstream DNA sequence on a replicative plasmid (pMF564). After complementation of the mutant strain, the biofilm formation ability was restored to wild-type levels (Fig. 4), indicating that the biofilm defect was indeed a result of the *Bcam1349* deletion.

Conclusion. The Gateway-compatible allelic exchange system described here takes advantage of bacteriophage lambda-based site-specific recombination instead of the traditional cloning procedures based on restriction enzymes and ligase and provides flexibility and efficiency. With proper primer design, the system allows precise in-frame deletion of open reading frames without generating truncated genes, reducing the risk of undesired polar effects. Moreover, the unmarked nature of the deletion procedure enables repetitive rounds of gene deletions in a single strain. We believe that the allelic exchange system described here will be useful in understanding the genetic basis of virulence in *B. cenocepacia* and in systematic analyses of the functions of genes in the physiology of this emerging pathogen and other *Burkholderia* species with medical relevance or potential biotechnological use. Furthermore, this allelic exchange system may enable the engineering of *Burkholderia* strains that retain their biotechnologically useful functions but are attenuated for virulence.

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