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

Mustafa Fazli, Joe J. Harrison,Michela Gambino,Michael Givskov, Tim Tolker-Nielsen

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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 *Beam1349* gene, encoding a cyclic di-GMP (c-di-GMP)-responsive regulator protein important for biofilm formation.

*B. 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 phenylalanine tRNA synthase (14) have been used in some *Burkholderia*...
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 gene deletion is their dependence on restriction and ligation enzymes for cloning. Restriction-free cloning based on Gateway 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 phsE-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 indicate 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-phaeS, 0.1% (wt/vol) p-chlorophenylalanine (pCp) (1α-4-chlorophenylalanine; Sigma-Aldrich) was autoclaved together with B. cenocepacia DB3.1, which contains an aprA62 mutation (Invitrogen).

### 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-Scel-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-phaE, pEX18Gm-phaE, and pEX18Km-phaE (14), resulting in the allelic exchange vectors pDONRPEX18Tp-Scel-phaE, pDONRPEX18Gm-Scel-phaE, and pDONRPEX18Km-Scel-phaE, 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 gyrA62 mutation (Invitrogen).

### Construction of the I-SceI expression vector pDAI-SceI-phaeS. 

To construct pDAI-SceI-phaeS (Fig. 1), an ~1.2-kb fragment containing the phaE gene was excised from pUC57-phaE (14) by restriction with XbaI and...
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-Sce1-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-attB1 and GW-attB2 (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

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; ccdR, 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\r\nS12, 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-Sce1-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.
TABLE 2 Primers used in this study

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<tr>
<th>Primer</th>
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<tr>
<td>Gene specific</td>
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<tr>
<td>Bcam1349-UpF and GWL</td>
<td>TACAAAAAAGCAGGCTAACGGGGATTTCGCACGAT</td>
</tr>
<tr>
<td>Bcam1349-UpR and tail</td>
<td>GGCACGTGACTGCACTGCAAGCTCAGGATGAGATTGATCGCCGACAT</td>
</tr>
<tr>
<td>Bcam1349-DnF</td>
<td>TACAGAAGGCTTGGTGTGAAAAGCAGGCT</td>
</tr>
<tr>
<td>Bcam1349-DnR and GWR</td>
<td>TACAGAAGGTCTGGGTGAAAAGCAGGCT</td>
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Common†                  |                                  |
| GW-attB                 |                                  |
| GW-attB                 |                                  |
| Amplification of the Gateway donor site |                              |
| GWE-SceI-F and GWE-R    | TACTACAAGCATTAGGGATAAACAGGTATAGCATGGATGTTTTCCCATG |
|                         | TACACTCTAGATCAGAAGATTTGGAGACACGGG |

Other†                  |                                  |
| Bcam1349-F             | TACTACCCCCGGGTAAATCGCTATTTCCGGCTG |
| Bcam1349-R             | TACTACTCTAGAATCGATATTTTCGACGGCAGATTGATCGCCGACAT |
| Bcam1349-RBS-F         | TACTACCCCCGGGTAAATCGCTATTTCCGGCTG |
| Bcam1349-RBS-R         | TACTACTCTAGAATCGATATTTTCGACGGCAGATTGATCGCCGACAT |

† Double-underlined sequences are common for all genes amplified and overlap the GW-attB primer sequences (29).
‡ The sequence in boldface type overlaps the gene-specific DnF primer.
§ Sequences were obtained from reference 29.
¶ Restriction enzyme sites are underlined.
© Restriction sites are underlined.

Primers GW-attB1 and GW-attB2 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-attB1 and GW-attB2 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 recombinatorial transfer of the mutant allele into the allelic exchange vector pDONRPEX18T-p-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 of Tp ml⁻¹ were screened by colony PCR using primers GW-E-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 of 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 sette was excised from plasmid pYedQ (21) by restriction with BamHI and HindIII. The presence of the insertion HindIII was inserted 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 recycling 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 Smal and XbaI restriction sites, respectively. The PCR fragment was digested with Smal 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 pENTRPEX18T-p-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 of 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 of Tet ml⁻¹. Batches of 10 Tp-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 of AB-glucose medium containing 0.1% (wt/vol) C specifically 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 pEX18T-ppheS, pEX18Gm-pheS, and pEX18Km-pheS, which contain different antibiotic resistance markers, were first described by Barrett and colleagues (14). These vectors are derivatives of pEX family vectors (25), in which the counterselectable marker gene was replaced with a mutant allele of the B. pseudomallei pheS gene. Here we modified...
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/H11001 host cells, such as E. coli DH5/H9251 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 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 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.
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 down-stream homology regions of the target gene. 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-attB1 and GW-attB2 (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
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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REFERENCES


