TECHNICAL ADVANCE

Knockout of a highly GC-rich gene in *Burkholderia pyrrocinia* by recombineering with freeze-thawing transformation

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Abstract

Genetic transformation is a valuable and essential method that provides powerful insights into the gene function of microorganisms and contributes to the construction of engineered bacteria. Here, we developed a novel genetic transformation system to easily knock out a highly GC-rich gene (74.71% GC) from *Burkholderia pyrrocinia* JK-SH007, a biocontrol strain of poplar canker disease. This system revealed a reliable selectable marker (trimethoprim resistance gene, *Tmp*) and a simplified, efficient transformation method (6,363.64 CFU/µg, pHKT2) that was developed via freeze-thawing. The knockout recombineering of *B. pyrrocinia* JK-SH007 was achieved through a suicide plasmid with a three-fragment mutagenesis construct. The three-fragment cassette for mutagenesis was generated by overlap extension and touchdown PCRs and composed of *Tmp* flanked by GC-rich upstream and downstream fragments from *B. pyrrocinia* JK-SH007. The mutant strain ($\Delta BpEG$), which was verified by PCR, lost 93.3% of its ability to degrade carboxymethyl cellulose over 40 days. Overall, this system may contribute to future research on *B. pyrrocinia* traits.

KEYWORDS

Burkholderia pyrrocinia, freeze-thawing transformation, GC-rich gene, homologous recombination, knockout

1 | INTRODUCTION

The ability to manipulate the bacterial chromosome in molecular genetics and genetic engineering studies can provide significant insight into gene function (Fan et al., 2016; Ito et al., 2005). In vivo analysis of gene function and construction of the engineered bacteria require reliable genetic transformation (Cao et al., 2017; Matheka et al., 2019; Riglar & Silver, 2018). An optimal genetic transformation system generally includes an efficient transformation system, a reliable gene-editing system, and a simplified screening method (Niyomtham et al., 2018; Rivera et al., 2014; Silva et al., 2016; Zeaiter et al., 2018). Recently, *Burkholderia* species have gained research interest as they exhibit potential for biological control. As an endophytic bacterium, *Burkholderia pyrrocinia* has been used as a biocontrol bacterium to control plant diseases (Arrielelias et al., 2019; Kwak & Shin, 2015; Lee et al., 2012; Ren et al., 2011) and promote plant growth (Madhaiyan et al., 2010; Ren et al., 2011). For example, *B. pyrrocinia* JK-SH007 can be used for biocontrol of poplar canker to promote the growth of poplar (Ren et al., 2011). However, there are limited studies on the mechanisms of *B. pyrrocinia*-mediated biocontrol of pathogenic fungi in plants. One of the key reasons for slow research development could be the lack of an effective genetic transformation system.

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One of the essential considerations in genetic transformation is the effective transfer of foreign DNA into the recipient *Burkholderia*. Heat-shock transformation can be used to obtain high efficiency transformation in *Escherichia coli* (Singh et al., 2010), but this method is rarely reported to be suitable for the transformation of *Burkholderia*. Moreover, electroporation-based transformation methods for *Burkholderia* are limited because they require sophisticated, expensive equipment and supplies (Kang et al., 2011; Li et al., 2019). Although the transformation of *Burkholderia pseudomallei* 4845 by electroporation was successful (Mack & Titball, 1996), Tomlin et al. (2004) showed that five species from *Burkholderia* cepacian complex were not suited for electroporation as a means of introducing plasmids. Therefore, freeze-thawing-based transformation (Lai et al., 2009) was explored as an alternative for electroporation for further genetic manipulation of *B. pyrrocinia* JK-SH007.

Recently, multiple mutagenesis strategies were harnessed to manipulate genomic DNA, which mainly involved CRISPR-Cas9 (Cong et al., 2013) and homologous recombination (HR) (Sharan et al., 2009). The HR-based mutagenesis strategy is still one of the most effective ways to manipulate bacterial genes, which relies on recombinase systems and a homologous mutagenesis cassette (Murphy et al., 2000; Sharan et al., 2009). However, genome manipulation of *Burkholderia* remains challenging, as described by Wang et al. (2018) and Kang et al. (2011). In particular, the Red α /Red β recombinase system from λ phage, an HR-based catalysis strategy, was not functional in some *Burkholderia* species (Barrett et al., 2008; Kang et al., 2011).

Interestingly, the genomic DNA of *Burkholderia* contains GC-rich sequences (Kang et al., 2011; Teng et al., 2017). In particular, the GC content of *B. pyrrocinia* is more than 65% (Kwak & Shin, 2015). The proportion of GC in some genes of *B. pyrrocinia* is as high as 75% (Chen, Ye, Sista Kameshwar et al., 2020). This high-GC content DNA leads to difficulties in the amplification of large PCR products and the construction of the mutagenesis cassette (Hube et al., 2005; Kang et al., 2011). Meanwhile, high-GC DNA presents a potential challenge for the mutagenesis of *Burkholderia* (Koh et al., 2014). In addition, the lack of reliable and stable selection markers affects mutagenesis in *Burkholderia*, therefore there are limited reports on gene editing in *Burkholderia*.

Genetic transformation of *B. pyrrocinia* is crucial for determining the functions of genes. Herein, we present a selectable marker, suitable vector, efficient transformation system, reliable mutagenesis strategy for GC-rich genes, and simplified methods for detecting gene knockouts.

2 | RESULTS

2.1 | Selection of a resistance gene for *B. pyrrocinia* JK-SH007

To establish a simplified and efficient genetic transformation system for *B. pyrrocinia* JK-SH007 (Table 1), the first step was finding a reliable selectable marker. The antibiotic susceptibility test was performed to find reliable resistance genes for the selection of TABLE 1 Bacterial strains and plasmids used in this study

	Description	Source or reference
Strain		
Burkholderia pyrrocinia JK-SH007	A potential biocontrol agent for poplar canker	Ren et al. (2011); CCTCC no. M209028
Burkholderia multivorans WS-FJ9	A plant growth-promoting rhizobacterium	Li et al. (2013); CCTCC no. M2011435
Burkholderia cenocepacia NSM-05	A potential biocontrol agent for pine shoot blight disease	Wu and Wu (2014); CCTCC no. M2011015
Escherichia coli DH5α	F-φ80 lacZΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17(rk ⁻ ,mk ⁻)supE44 λ thi-1 gyrA96 relA1 phoA	Our laboratory
E. coliDH5α _{λpir}	RP4-2-tet: Mu-kan::Tn7 integrant leu-63::IS10 recA1 creC510 hsdR17 endA1 zbf-5 uidA (Dmlul): pir + thi	Li et al. (2009)
Plasmid		
pMD19-T	Amp ^r , PCR cloning vector	From Takara
pHKT2	Tmp ^r , pBBR1Tp	Tomlin et al. (2004)
pUTTnsKm	Amp ^r , Km ^r , sacB, sacBDR, oriR6K, oriTRP4, <i>tnp</i>	Li et al. (2009)
Tmp-pMD19-T	Amp ^r , Tmp ^r	This study
pUT-eUTD	Amp ^r , Tmp ^r , oriR6K,	This study
pMD-19-UTD	Amp ^r ; Tmp ^r , PCR cloning vector	This study

Note: CCTCC, China Center for Type Culture Collection.

the genetic transformation of *B. pyrrocinia* JK-SH007. The results (Table S1) showed that the antibiotics ampicillin (Amp), tetracycline (Tet), spectinomycin (Spe), streptomycin (Str), and lincomycin (Lin) had almost no inhibitory effect on *B. pyrrocinia* JK-SH007, even if the working concentration was increased to 300 μ g/ml. Furthermore, *B. pyrrocinia* JK-SH007 showed a certain sensitivity to rifampicin (Ra) and erythromycin (Em) at 24 hr but became insensitive after 48 hr.

However, *B. pyrrocinia* JK-SH007 exhibited sensitivity to trimethoprim (Tmp), which could effectively inhibit *B. pyrrocinia* JK-SH007 (20 μ l, overnight culture) at 50 μ g/ml on a plate. There was no colony growth within 72 hr, while the control produced colonies at 24 hr. Therefore, the Tmp-resistance gene can be used as a selection marker for the genetic transformation of *B. pyrrocinia* JK-SH007.

Furthermore, chloramphenicol (Cm) and kanamycin (Kan) showed unstable inhibitory effects. When the concentration of Cm and Kan reached 300 μ g/ml, *B. pyrrocinia* JK-SH007 showed very little growth. Thus, the concentrations of Cm and Kan were further increased for subsequent experimental analysis. *B. pyrrocinia* JK-SH007 showed sensitivity when the Cm concentration reached 350 μ g/ml and when the (a)

(C)

Kan concentration reached 450 µg/ml. Nevertheless, there was an approximately 50% probability of obtaining 1 to 20 negative colonies on the plate. Therefore, Cm and Kan can be used as potential antibiotics in transformation tests and resistance studies of *B. pyrrocinia* JK-SH007.

2.2 | Different transformation methods in **B.** pyrrocinia JK-SH007

B. pyrrocinia JK-SH007 was subjected to heat-shock and freezethawing transformation. The plasmid pHTK2 is a vector that contains the Tmp resistance gene that encodes a Tmp-resistant dihydrofolate reductase; it has a broad-host-range origin of replication and is capable of being maintained in Burkholderia. The plasmid pHKT2 (Table 1) was transferred to *B. pyrrocinia* JK-SH007 as a means of exploring the ability of the strain to be transformed with DNA. The freeze-thawing transformation was approximately 6- to 8-fold more efficient than the heat-shock transformation (Figure 1a,b), while the heat-shock transformation was unstable and often failed to transform the cells (three failures in eight tests). The results showed that pHKT2-JK-SH007 could generally grow on plates containing Tmp, while wild-type B. pyrrocinia JK-SH007 could not (Figure 1c,d). Furthermore, the Tmp resistance gene (*Tmp*) was also detected on positive clones by colony PCR. The results revealed that the plasmid pHKT2 was efficiently transferred into B. pyrrocinia JK-SH007 using freeze-thawing transformation with Tmp as a reliable selection marker.

2.3 | Effect of the plasmid amount on transformation

The effect of the amount of plasmid used on transformation was explored. Different amounts of the pHKT2 were used for transformation, as shown in Figure 1e. When 10 ng and 20 ng of plasmid were used, the transformation efficiency fluctuated greatly. When 20-90 ng of plasmid was used, the transformation efficiency generally showed an upward trend, peaking at 90 ng. Once the amount of plasmid exceeded 500 ng, the transformation efficiency began to decrease before becoming stable. This may be due to plasmids reaching a saturation state, resulting in their inability to continue to improve the transformation efficiency.

2.4 Effect of the growth state of competent cells on transformation



n

0.0

FIGURE 1 The transformation of Burkholderia pyrrocinia JK-SH007 using plasmid pHKT2. (a) The heat-shock transformation. The plasmid pHKT2 was transferred into B. pyrrocinia JK-SH007 by the heat-shock transformation method. (b) The freeze-thawing transformation. The plasmid pHKT2 was transferred into B. pyrrocinia JK-SH007 by the freeze-thawing transformation method. (c) Testing the transformation on a plate containing trimethoprim (Tmp). Left, B. pyrrocinia JK-SH007; right, B. pyrrocinia JK-SH007 containing plasmid pHKT2. (d) Testing of the transformation on plate containing ampicillin. Left, B. pyrrocinia JK-SH007; right, B. pyrrocinia JK-SH007 containing plasmid pHKT2. (e) The effect of the concentration on transformation. (f) The effect of the OD₆₀₀ of competent cells on transformation. All experiments were performed in three biological replicates. Data are presented as means of three replicates \pm SD

The effect of the growth state of competent cells on transformation was also explored using competent cells at various optical densities

0.4

0.2

0.6

Competent Cells (OD₆₀₀)

0.8

1.0

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at 600 nm (OD₆₀₀ values), including 0.1, 0.3, 0.5, 0.7, and 0.9. The transformation efficiency (cfu/ μ g) = number of colony-forming units (cfu)/plasmid quantity (μ g).

The transformation rates of the competent cells at OD_{600} values of 0.1, 0.3, 0.5, 0.7, and 0.9 were 54, 911, 1,016, 101, and 94 cfu/µg, respectively. The transformation efficiency reached a peak of 1,016 cfu/µg at an OD_{600} of 0.5 (Figure 1f).

2.5 | Optimization of the transformation system by an orthogonal assay

Optimization of the transformation system of B. pyrrocinia JK-SH007 was carried out using an orthogonal experimental design according to the Taguchi method (Jeyapaul et al., 2005). In the present study, all selected factors were examined using an orthogonal L25(5)⁶ test design (Table 2). The results of the tests presented in Table S2 indicate that the maximum transformation efficiency was 6,363.6 cfu/ μ g. The range analysis showed that the influence on the mean transformation efficiency decreased in the order: competent cells (OD_{600}) > thawing temperature > incubation (on ice) time > thawing time > plasmid amount > freezing time according to the R values. Analysing the variance revealed that the six factors showed significant differences in the transformation efficiency of B. pyrrocinia JK-SH007 (Table S3). The OD_{600} value of competent cells was the main factor affecting transformation efficiency. The maximum transformation efficiency was obtained by the optimized method, where 110 ng of pHKT2 plasmid was added into the competent cells of B. pyrrocinia JK-SH007 at an OD_{600} of 0.5. The cells were incubated for 75 min on ice, frozen for 60 s in liquid nitrogen, and thawed for 1 min at 40 °C in water, as described in detail in the Experimental Procedures.

2.6 | Extended application of the improved transformation system in other *Burkholderia* species

The optimized procedure was expanded to other species of *Burkholderia* to further test the reliability of the transformation strategy. The plasmid pHKT2 was successfully transferred into *Burkholderia multivorans* WS-FJ9 and *Burkholderia cenocepacia*

NSM-05 using the optimized procedure. *B. pyrrocinia* JK-SH007 exhibited the highest transformation efficiency (100%, 6,363.6 cfu/ μ g), followed by *B. cenocepacia* NSM-05 (77%, 4,883.4 cfu/ μ g), and *B. multivorans* WS-FJ9 (43%, 2,736.1 cfu/ μ g). This demonstrates that the optimized procedure is a reliable method that has the potential to be applied to other species of *Burkholderia*.

2.7 | Overview of the knockout strategy

A novel knockout strategy for B. pyrrocinia JK-SH007 was developed, as described in Figure 2. The target gene was replaced by a suicide plasmid through HR, which relied on the host's recombinase system. The upstream (U) and downstream (D) regions of the target gene were amplified and fused on either side of the *Tmp* gene (T) to generate the three-fragment mutagenesis cassette (UTD). The UTD fragment was cloned into the pMD19-T vector, and the resultant vector was named pMD19-UTD (Figure 3a). A new suicide plasmid was constructed by HR cloning (Figure 3b). The knockout plasmid pUT-UTD was derived from pUTTns (Li et al., 2009), which included the element of *oriR6K* and UTD fragment for editing of target genes (Figure 3c). Afterwards, the plasmid pUT-UTD was transferred into B. pyrrocinia JK-SH007 using freeze-thawing. Then, the target gene was knocked out using the suicide plasmid pUT-UTD through HR via the host's recombinases. Subsequently, the candidate transformants were screened using a carefully designed PCR detection process. To verify positive knockout, specific PCR primers were used for the Tmp gene (e.g., primer pair A; Table 1), the upstream detection fragment (external Up-Up-Tmp internal), and the downstream detection fragment (Tmp internal-Down-external Down) (e.g., primer pair H, primer pair I; Table 1).

2.8 | Preparing the three-fragment mutagenesis cassette with GC-rich DNA from *B. pyrrocinia*

The three-fragment cassette for HR was constructed as described in detail in the Supporting Information. Genome sequencing analysis of *B. pyrrocinia* revealed that the genomic DNA was rich in GC regions (Kwak & Shin, 2015). The endo-1,4- β -glucanase gene (*BpEG*) (74.71% GC) was chosen as the target gene, which can cleave carboxymethyl

 TABLE 2
 Design of orthogonal L25(5)⁶ test

	Test factors							
Level	Incubation time (min)	Freezing time (sec)	Thawing time (min)	Plasmid amount (ng)	Thawing temperature (°C)	Competent cells (OD ₆₀₀)		
1	15	30	1	70	32	0.1		
2	30	60	2	80	35	0.3		
3	45	90	3	90	37	0.5		
4	60	120	4	100	40	0.7		
5	75	150	5	110	42	0.9		



FIGURE 2 Knockout recombineering strategy. The target gene was replaced by a suicide plasmid through homologous recombination (HR) that relied on the host's recombinase system. The example shown here involves the amplification of GC-rich genes, construction of three-fragments and suicide plasmid, HR, and detection by PCR. The upstream (U) and downstream (D) region of the target gene were amplified and fused on the upstream and downstream sides, respectively, of the trimethoprim (*Tmp*) resistance gene (T) to generate the three-fragment mutagenesis cassette (UTD). The knockout plasmid pUT-UTD was constructed by HR cloning, which included the element of *oriR6K* and UTD fragment for editing of target genes. The GC-rich gene was knocked out by recombineering using pUT-UTD with freeze-thawing transformation. Subsequently, the candidate transformants were screened using a PCR detection process

cellulose (CMC) into glucose, as we previously reported (Chen, Ye, Sista Kameshwar et al., 2020). The three-fragment cassette was constructed by overlap-extension PCR combined with touchdown PCR instead of the classic digestion-ligation system (Figure 4a). The upstream and downstream fragments of the target gene were amplified and fused on either side of the *Tmp* gene to generate the threefragment mutagenesis cassette.

First, the upstream and downstream fragments of the *BpEG* gene were obtained by PCR1 and PCR3 (Figure 4b), while the

resistance gene with the promoter (*Tmp*) was amplified by PCR2 (Figure 4b). The PCR1-generated upstream fragment (with primer pair B) included 18–25 bases at the 3' end overlapping with the 5' end of *Tmp*. Similarly, 18–25 bases were added to the PCR3-generated downstream region (primer pair C) at the 5' end overlapping with the 3' end of *Tmp*. The resistance gene (*Tmp*) (PCR2, primer pair D) was also flanked by 18–25 bases of the upstream and downstream sequences. Therefore, the overlap region of the junctions increased from 25–50 bp to 36–50 bases, which facilitated



FIGURE 3 Genetic constructs for knockout recombineering in *Burkholderia pyrrocinia*. (a) The structure of the plasmid pMD19-UTD containing three-fragment, *ori*, and other elements. The plasmid pMD19-UTD is maintained in *Escherichia coli* DH5 α . (b) The construction of suicide plasmid by recombinase using 20–25 bp homology sequence. (c) The structure of the suicide plasmid pUT-UTD containing three-fragment cassette and R6K π origin. The plasmid is used to knock out the target gene and is maintained in *E. coli* DH5 $\alpha_{a,nir}$ (Li et al., 2009)

the construction of the three-fragment mutagenesis cassette (Figure 4a and Table 3). In contrast, if the overlap region had been only 18–25 bp, there would have been an increased risk of experimental failure in the construction of a GC-rich three-fragment mutagenesis cassette.

Second, the touchdown PCR (PCR4) was performed using a mixture of the products from PCR1, PCR2, and PCR3 (Figure 4c). This process was the critical step for the successful construction of the three-fragment mutagenesis cassette to prevent experimental failure. Third, the PCR5 was performed using the product of PCR4 as a DNA template, whose product was analysed using a 1% agarose gel. The results showed that the three-fragment cassette (Up + *Tmp* + Down, eUTD) was successfully constructed (Figure 4d). The resulting 2.7-kb DNA fragment was purified and cloned into the pMD19-T vector (Takara Bio) and the recombinant plasmid was named pMD-19-eUTD.

2.9 | Effects of different DNA forms on gene knockout

Four DNA forms were transformed into *B. pyrrocinia* JK-SH007 by the transformation method mentioned above to achieve HR (Figure 4e). These forms included the three-fragment of HR (eUTD) (Figure 4f), truncated HR fragments (eUT' and eT'D) (Figure 4g), the plasmid pMD-19-eUTD (Figure 4h), and the suicide plasmid pUT-eUTD (Figure 4i). Mutagenesis was attempted with the fragments,

which included single-crossover mutagenesis (eUT' and eT'D) and double-crossover mutagenesis (eUTD). However, these attempts failed to achieve the knockout recombineering of the GC-rich gene from *B. pyrrocinia* JK-SH007. The analogous results showed that there were no positive clones obtained by mutagenesis of the plasmid pMD-19-eUTD. This indicates that fragment-based and plasmid pMD-19-eUTD-based transformations for knockout cannot be applied to *B. pyrrocinia* JK-SH007.

Furthermore, a new suicide plasmid, pUT-eUTD derived from pUTTns, was constructed, and this plasmid included the R6K origin of replication and the eUTD fragment for editing *BpEG*. The *BpEG* gene was knocked out through HR using the suicide plasmid pUT-UTD with the assistance of the host recombinase system. PCR examination showed that positive clones were present on a plate containing Tmp, as described in Figure 5, and the HR frequency was 94.9%. These results indicate that we identified a suitable form of DNA for *B. pyrrocinia* JK-SH007 knockout and revealed a strategy to knock out highly GC-rich genes.

2.10 | Detection of the gene knockout by PCR and Southern blotting

Confirmation of the knockout was conducted using a PCR detection process designed based on the results shown in Figure 5a. The *Tmp* gene of the clones was first detected by PCR using primer pair A. Then colonies 2, 3, and 5 shown in Figure 5b were selected for



FIGURE 4 Preparing for the knockout. (a) Strategy for the construction of three-fragment cassette of BpEG gene. The upstream (U) and downstream (D) regions of the target gene were amplified and fused on the upstream and downstream sides, respectively, of the trimethoprim (Tmp) resistance gene (T) to generate the three-fragment mutagenesis cassette (UTD). (b) Amplifications of the Up fragment (PCR1), Tmp gene (PCR2), and Down fragment (PCR3). (c) The products (PCR4) of touchdown PCR. The equal volumes of the products of PCR1, PCR2, and PCR3 were performed with the annealing temperature decreasing from 68 to 60 °C at 1 °C/cycle. (d) Amplification of three-fragment cassette (PCR5) by overlap-extension PCR combined touchdown PCR. (e) Strategy for the transformation of four different DNA constructs into Burkholderia pyrrocinia JK-SH007. (f) The three-fragment cassette (eUTD). (g) The truncated fragments (eUT' and eT'D). (h) The plasmid pMD-19-eUTD. (i) The suicide plasmid pUT-eUTD

further testing by PCR using the primer pairs H and I. The upstream detection fragment (external Up-Up-Tmp internal) was tested using primer pair H, while the downstream detection fragment (Tmp internal-Down-external Down) was tested using primer pair I. The molecular weights of the PCR products were 2,400 bp (Figure 5c, primer pair H) and 1,600 bp (Figure 5d, primer pair I) by analysis of agarose gel electrophoresis, which is consistent with the design. Additionally, the knockout was further confirmed by Sanger sequencing. These results showed that the BpEG gene was successfully knocked out at the designed position, while the possibility of knockout at other positions was ruled out, suggesting that this design has greater potential in the detection of the gene knockout instead of Southern blotting.

Furthermore, the Southern blotting result showed that Tmp was detected in the genomic DNA of the mutant B. pyrrocinia JK-SH007 $(\Delta BpEG)$ but not in the genomic DNA of the wild type (Figure 5e). This result further confirmed that the BpEG gene was successfully knocked out. Meanwhile, the result proved that only one copy of the BpEG gene was knocked out.

2.11 | Analysis of biological properties

To further prove that the BpEG gene was knocked out, the ability to degrade CMC was determined in wild-type and mutant strains of B. pyrrocinia JK-SH007 using Congo red staining on agar plates

TABLE 3 The oligonucleotides used in this study

No.	Primer name	Sequence (5'-3')	Tm	Primer pairs (no. primer, annealing temperature)	
1	Tmp-F	AATTCACGAACCCAGTTGACA	55.0	A (1 + 2, 55 °C)	
2	Tmp-R	TTAGGCCACACGTTCAAGTG	55.5	B (3 + 4, 68 °C)	
3	U-EG-F	GCGCAAGCTGCTCGTCGTCA	63.4	C (5 + 6, 66 °C)	
4	U-EG-R	GTCAACTGGGTTCGTGAATTAACCCCCGCCATCCGTTACA	70.2	D (7 + 8, 55 °C)	
5	D-EG-F	CACTTGAACGTGTGGCCTAAAGTGCGCCGCGCGCATGTC	75.2	E (9 + 10, 50 °C)	
6	D-EG-R	CGCCATCGCCTCCGTATGGT	61.8	F (11 + 12, 62 °C)	
7	E-Tmp-F	TGTAACGGATGGCGGGGGTTAATTCACGAACCCAGTTGAC	70.2	G (15 + 16, 52 °C)	
8	E-Tmp-R	GACATGCGGCGCGCGCACTTTAGGCCACACGTTCAAGTG	75.2	H (15 + 14, 55 °C)	
9	Simple 1	GATCTGCAGGTGGCACTTTTC	52.7	I (13 + 16, 58 °C)	
10	Simple 2	AATTCCACAAATTGTTATCCGC	56.7	J (3 + 6, 68-62 °C, 62 °C)	
11	SP-EG-F	GAAAAGTGCCACCTGCAGATCGCGCAAGCTGCTCGTCGTCA	74.1		
12	SP-EG-R	CGGATAACAATTTGTGGAATTCGCCATCGCCTCCGTATGGT	69.2	K (3 + 14, 68-63 °C; 62 °C)	
13	Tmp-Tr-F	GTATGCGCTCACGCAACTGGTCC	63.1	L (13 + 6, 68-63 °C;	
14	Tmp-Tr-R	GAGCCTGGGTGGGATTCGGAC	66.7	62 °C)	
15	C-EG-F	GCGCTATTTCTCGGATTT	50.3		
16	C-EG-R	GCCAGGCTCTGCCATTT	55.0		

CHEN ET AL.

containing 1.5% CMC. The Congo red plate test showed that the mutant strain ($\Delta BpEG$) almost entirely lost the ability to degrade CMC and exhibited minimal ability to degrade CMC for survival (Figure 5f), further proving that the *BpEG* gene was knocked out.

To further evaluate the genetic stability after the knockout, the strains were observed for 40 days. Over this duration, the mutant strain ($\Delta BpEG$) showed minimal ability to degrade CMC for survival, while the wild type continued to release reducing sugars (Figure 5g). It was found that the knockout strain ($\Delta BpEG$) lost 93.3% of its ability to degrade CMC (0.038 mg/ml) compared to the wild-type strain (0.57 mg/ml) over 40 days. Overall, the knockout strain exhibited genetic stability when the *BpEG* gene was knocked out using the genetic transformation system.

3 | DISCUSSION

Effective transformation (Ohmine et al., 2016) and genetic manipulation (Koh et al., 2014) of GC-rich genes are associated with reliable selectable markers, efficient transformation systems, and precise gene-editing systems. In this study, we established and optimized the transformation system of *B. pyrrocinia* JK-SH007 while achieving knockout of a highly GC-rich gene (Figure 6).

The antibiotic susceptibility tests revealed that Tmp could inhibit *B. pyrrocinia* JK-SH007 (20 μ l, overnight culture) at low concentrations (50 μ g/ml) on the plate, which was supported by preliminary experiments (100%, 12 tests). Similarly, Tomlin et al. (2004) showed that *Burkholderia* cepacian complex strains were susceptible to Tmp, which included *B. cepacia* CEP509, *B. multivorans* ATCC

17616, *B. cenocepacia* K56-2, *Burkholderia stabilis* LMG 14086, and *Burkholderia vietnamensis* 16232. Therefore, the *Tmp* resistance gene is a reliable selection marker that can contribute to the study of transformation and genetic modification.

Moreover, Cm and Kan can also be used as alternative antibiotics. However, there are potential risks including the requirement of high concentrations and instability (approximately 50% probability of 1–20 negative colonies on a plate). It is possible that *B. pyrrocinia* JK-SH007 contains the resistance genes for these antibiotics or other alternative pathways that degrade Cm and Kan. Similarly, it was proven that *B. pseudomallei* can encode resistance to kanamycin, as described by Hamad et al. (2009). Generally, the antibacterial mechanism of Cm and Kan is that they bind to the ribosome of the bacteria (Cm binds to the 50S subunit, Kan binds to the 30S subunit) to inhibit protein synthesis. However, *B. pyrrocinia* JK-SH007 could produce glycocalyx (Chen, Ye, Chio et al., 2020) and biofilm (Fu et al., 2020), which may limit the entry of Cm and Kan into the cell and affect the function of Cm and Kan.

The highest transformation efficiency of *B. pyrrocinia* JK-SH007 obtained by the freeze-thawing transformation method was 6,363.6 cfu/ µg, which is approximately 20-fold higher than that obtained by the traditional heat-shock method. The efficiency of the improved method was also higher than the efficiency (250 cfu/µg) obtained by electroporation of *B. pseudomallei* 4845 using frozen cell suspensions, as described by Mack and Titball (1996). Moreover, the scope of application of the transformation method was expanded. The results showed that pHKT2 was successfully transferred into *B. multivorans* WS-FJ9 (2,736.1 cfu/µg) and *B. cenocepacia* NSM-05 (4,883.4 cfu/µg), and the efficiency was similar to the maximum efficiency (1,000 cfu/µg) obtained for *Bacillus anthracis* using the freeze-thawing transformation



FIGURE 5 The detection of deletion mutants (BpEG) of Burkholderia pyrrocinia JK-SH007. (a) Strategy for BpEG deletion by PCR. (b) The detection by PCR using primer pair A. (c) The detection by PCR using primer pair H. (d) The detection by PCR using primer pair I. (e) Southern blotting test. The purified fragment of Tmp gene was labelled as a probe. Southern blotting was performed using digoxigenin (DIG) High Prime DNA Labeling and Detection Starter Kit I. (f) The Congo red plate test. Both wild-type and mutant B. pyrrocinia JK-SH007 strain were dropped on the carboxymethyl cellulose (CMC) agar plate containing 0.7% agar. All plates were incubated at 28 °C for 5 days and stained with 0.1% (wt/vol) Congo red solution for 30 min and visualized by washing with 1 M sodium chloride solution for 10 min. (g) The genetic stability tests. The mutant strain ($\Delta BpEG$) showed minimal ability to degrade CMC for survival, while the wild type continued to release reducing sugars over 40 days. The hydrolysis ability was calculated according to the method of Guo et al. (2017)

method (Stepanov et al., 1990). Therefore, our improved transformation system has great potential and can be very useful in experiments to manipulate the Burkholderia genome.

Notably, this study demonstrated a concise and cost-effective protocol for amplifying highly GC-rich fragments and provided an alternative method for GC-rich gene colony PCR, as described in

the Experimental Procedures section. Moreover, our study demonstrated the procedure for constructing a three-fragment mutagenesis cassette with high-GC content, which required overlap extension PCR combined with the touchdown PCR. Although overlap extension PCR is commonly used to construct three-fragment mutagenesis cassettes (Wang et al., 2020), the amplification was hampered by the



FIGURE 6 The transformation and recombineering scheme for the knockout in *Burkholderia pyrrocinia* JK-SH007. Strategy for transformation is shown on the left; the right section presents the strategy for knockout recombineering and detection. Detailed descriptions for each step are provided in the study

size and GC content of target DNA (Kang et al., 2011). This may be one of the reasons why there are few reports on the knockout of highly GC-rich genes. However, the touchdown PCR (PCR4,5 procedures) greatly improved this situation. In addition, extending the homologous sequence (from 20 to 50 bp) on the junction facilitated the construction of a three-fragment mutagenesis cassette during the GC-rich PCR process, similar to the strategy used for constructing mutagenesis cassettes (Yu et al., 2004). These results suggest that the procedures are very promising for manipulating GC-rich DNA sequences.

Generally, linear fragment-based mutagenesis is commonly used to manipulate genes in HR (Fang et al., 2018; Murphy et al., 2000; Wang et al., 2020; Yu et al., 2004). However, the results presented here demonstrated that linear fragment-based mutagenesis was not suitable for gene-editing of *B. pyrrocinia* JK-SH007. Analogous results showed that fragment-based transformation harbours a greater risk of knockout failure by HR (Datsenko & Wanner, 2000). It is possible that the foreign fragment DNA is unstable in vivo and vulnerable to attack by the B. pyrrocinia JK-SH007 immune system. Analogous reports have shown that foreign DNA is cleaved by endonuclease in bacterial and archaeal CRISPR immunity pathways (Jiang et al., 2013; Sashital et al., 2012). In addition, the results showed that mutagenesis was not functional using plasmid pMD-19-eUTD with an ori element, which enabled the plasmid to achieve a lot of copies in B. pyrrocinia JK-SH007. The ori element is essential for the initiation of DNA replication, as a specific sequence containing binding sites for DNA helicase. The combination of the helicase with the ori could promote the assembly of the DNA polymerase complex into a functional holoenzyme at the origin of the replication initiation site. Therefore, the DNA polymerase could be more functional than recombinase, resulting in the catalytic reaction of the recombinase to be limited. Furthermore, GC-rich DNA could be another source of failure as it makes HR difficult in cells. These reasons could all contribute to the limited reports on the mutagenesis of B. pyrrocinia.

Importantly, this study was the first to demonstrate the knockout of the BpEG gene (74.71% GC) from B. pyrrocinia JK-SH007 using a circular suicide plasmid by HR. We found that a plasmid with an $oriR6K\pi$ replication origin (Li et al., 2009) could not replicate in *B. pyrrocinia* JK-SH007. so the oriR6K element was selected to construct a suicide plasmid for mutagenesis in B. pyrrocinia JK-SH007. Compared with linear fragmentbased mutagenesis, the suicide plasmid pUT-eUTD may prevent or reduce the attack by the immune system of B. pyrrocinia JK-SH007. In addition, the stable characteristics of the suicide plasmid increase the probability of successful HR. Furthermore, compared with the plasmid pMD-19-eUTD-based mutagenesis, the recombinase was functional for HR by the suicide plasmid pUT-eUTD-based mutagenesis. The mutagenesis process was catalysed by the host recombinase in B. pyrrocinia JK-SH007. Similarly, Wang et al. (2018) discovered that recombinases in Burkholderia species enable genome mining. This is especially crucial for the mutagenesis of GC-rich genes. Taken together, the three PCR detection analysis and Southern blotting experiments indicated that the highly GC-rich genes of B. pyrrocinia could be knocked out using circular suicide plasmid by HR with the assistance of the host recombination system.

In summary, we developed a novel genetic transformation system, including a transformation system and knockout system for *B. pyrrocinia* JK-SH007. The transformation system efficiently transferred foreign plasmid DNA into *B. pyrrocinia* JK-SH007 using freeze-thawing. Furthermore, the highly GC-rich gene (74.71% GC) was knocked out using the suicide plasmid pUT-UTD through HR. Moreover, PCR technology was developed to detect gene knockouts. Overall, the genetic transformation system demonstrated stable transgene integration and transmission to the next generation.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and growth media

The list of bacterial strains and plasmids used in this study are provided in Table 1. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C. *B. pyrrocinia* JK-SH007, *B. multivorans* WS-FJ9, and *B. cenocepacia* NSM-05 were cultured in LB medium at 30 °C. The CMC medium (Chen, Ye, Susta Kameshwar et al., 2020) consisted of 1.5% carboxymethyl cellulose, 0.05% MgSO4.7H₂O, 0.1% NaNO₃, 0.1% KH₂PO₄, and 0.1% yeast extract.

4.2 | Antibiotic susceptibility testing of *B. pyrrocinia* JK-SH007

B. pyrrocinia JK-SH007 (Ren et al., 2011) was tested for antibiotic susceptibility using the resistance plate coating method. Twenty microlitres of a bacterial solution of *B. pyrrocinia* JK-SH007 cultured at 28 °C for 36 hr was coated on various kinds of resistance plates with different concentrations for different cultivation durations. The growth of the strains was observed and recorded after 24, 48, and 72 hr. All experiments were conducted in at least triplicate.

Molecular Plant Pathology 🚳

The following antibiotics were added when required at different concentrations (50, 100, 150, 200, 250, and 300 μ g/ml): Amp, Cm, Tet, Tmp, Kan, Spe, Em, Ra, Str, and Lin. Tmp was dissolved in *N*,*N*-dimethylacetamide at a concentration of 100 mg/ml. Cm was dissolved in ethanol at a concentration of 100 mg/ml. All other antibiotics were dissolved in sterile double-distilled water at different concentrations and stored at –20 °C until required.

4.3 | Different transformation methods for *B. pyrrocinia* JK-SH007

According to the results of antibiotic susceptibility testing, we chose the plasmid pHKT2 with Tmp resistance for transformation, as described in detail by Tomlin et al. (2004). The transformation of plasmid DNA into *B. pyrrocinia* JK-SH007 was explored by two methods. First, we used the traditional heat-shock method, as described by Singh et al. (2010), with slight modification. For the heat-shock treatment, 100 ng of the pHTK2 plasmid was mixed gently with 100 µl of competent cells. These cells were incubated on ice for 30 min, followed by a heat-shock treatment that included a 45 s heat-shock pulse at 42 °C, followed by 5 min of incubation on ice. Then, 950 µl LB medium was added to the tube and incubated at 30 °C for 3 h r at 225 rpm in a shaking incubator. The cultures were plated on LB plates containing Tmp and placed in an incubator at 28 °C for 36 hr.

We also explored the freeze-thawing transformation method, with slight modification, as described by Chen et al. (1994). In short, 100 ng of the pHTK2 plasmid was mixed gently with 100 μ l competent cells, and these cells were incubated on ice for 30 min and followed by a freeze-thawing treatment, which involved freezing for 60 s in liquid nitrogen and thawing for 2 min at 37 °C in water, followed by 5 min of incubation on ice. Then, 950 μ l of LB medium was added to the tube and incubated at 30 °C for 3 hr at 225 rpm in a shaking incubator. The cultures were plated on LB plates containing Tmp and placed in an incubator at 28 °C for 36 hr.

4.4 | Effect of the amount of plasmid and growth state of competent cells on transformation

The effect of the amount of plasmid on transformation was explored. The amounts of the pHKT2 plasmid used for transformation were 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500, 1,000, 1,500, and 2,000 ng. Various amounts of plasmid and 100 μ l of the competent cells of *B. pyrrocinia* JK-SH007 were gently mixed. The transformation was performed according to the procedure for the freeze-thawing transformation method mentioned above.

The various OD₆₀₀ values of competent cells used for the transformation were 0.1, 0.3, 0.5, 0.7, and 0.9. The next steps of the transformation were consistent with those mentioned above. The transformation efficiency (cfu/ μ g) was calculated as the number of colonies (cfu)/plasmid quantity (μ g). All experiments were conducted in at least triplicates.

Molecular Plant Pathology 👩

Competent cells of *B. pyrrocinia* JK-SH007, *B. multivorans* WS-FJ9, and *B. cenocepacia* NSM-05 were prepared with a kit according to the manufacturer's instructions (Competent Cell Preparation Kit; Takara). The prepared competent cells could be used for transformation experiments or stored at -80 °C for later use. Furthermore, an alternative strategy to prepare competent cells was developed. Briefly, 1-ml cultures were centrifuged at $1,500 \times g$ and 4 °C and the supernatant was discarded as soon as possible. Then, $100 \mu l$ of precooled 0.1 M CaCl₂ in ice was added to the microtube and the pellet was gently suspended. The microtube was centrifuged at $1,500 \times g$ and 4 °C, and the supernatant was treated with precooled 0.05 M CaCl₂ and 25% glycerin, and the pellet was gently suspended in the microtube.

4.5 | Optimization of the transformation system

An orthogonal L25(5)⁶ test design for the transformation model was used for the optimization of the transformation conditions. In our study, 25 transformations were carried out with incubation times of 15, 30, 45, 60, and 75 min on ice; freezing times of 30, 60, 90, 120, and 150 s; thawing times of 1, 2, 3, 4, and 5 min; thawing temperatures of 32, 35, 37, 40, and 42 °C; plasmid amounts of 70, 80, 90, 100, and 110 ng; and OD₆₀₀ values of competent cells of 0.1, 0.3, 0.5, 0.7, and 0.9. Table 1 shows the experimental conditions for the transformation of plasmid DNA into *B. pyrrocinia* JK-SH007. All experiments were performed in three biological replicates.

4.6 | Optimized transformation procedure for *B. pyrrocinia* JK-SH007

- 1. A total of 110 ng of the plasmid pHKT2 was added to 100 μ l of competent *B. pyrrocinia* JK-SH007 cells at an OD₆₀₀ of 0.5 and mixed by pipetting gently.
- 2. The tube in step [1] was incubated on ice for 75 min.
- The tube was frozen for 60 s in liquid nitrogen and thawed for 1 min at 40 °C in water.
- 4. The tube was transferred into ice for 5 min.
- 5. One millilitre of LB medium was added to the tube, then the cells were cultured in a shaker at 30 °C and 200 rpm for 3 hr.
- 6. The culture was concentrated to $200 \,\mu$ l by centrifugation at $10,000 \times g$ for 1 min and then plated on an LB plate containing 100 mg/ml Tmp. The plate was placed in an incubator at 28 °C for 36 hr.

4.7 | Exploration of the transformation of *Burkholderia* species by the improved procedure

The transformation of *B. multivorans* WS-FJ9 and *B. cenocepacia* NSM-05 was tested using the optimized transformation procedure. The transformation efficiency ($cfu/\mu g$) was calculated as mentioned above. All experiments were performed in triplicate.

4.8 | GC-rich gene PCR system

The GC-rich gene sequence obtained was amplified using a thermal cycler machine with the PCR mixture containing *B. pyrrocinia* JK-SH007 DNA (50–100 ng), 0.1 μ M of the specific primer (Table 3), 80 μ M of deoxynucleotide triphosphate (dNTP), 0.5 U LA *Taq* polymerase, and 10 μ l of 2 × GC buffer II (Takara Bio). The PCR was performed at the following conditions: 95 °C for 10 min; thermocycling consisting of 33 cycles at 95 °C for 30 s, annealing temperature (Table 3) for 30 s, and 72 °C for 1 kb/min; with a final extension at 72 °C for 10 min.

4.9 | Three-fragment mutagenesis cassette for homologous recombination

The three-fragment cassette for HR was constructed using overlapextension PCR combined with touchdown PCR technology, as described in the Supporting Information. First, both the upstream and downstream fragments of *BpEG* (Chen, Ye, Susta Kameshwar et al., 2020) were amplified by PCR (Table 3). These processes were named PCR1 (primer pair B) and PCR3 (primer pair C). The Tmp resistance gene (*Tmp*) with the promoter was also amplified by PCR using primer pairs A and D. The process using primer pair D was named PCR2. The PCR procedures for the GC-rich gene were similar, as described above. Second, we mixed equal volumes of the products of PCR1, PCR2, and PCR3. Touchdown PCR was performed with the annealing temperature decreasing from 68 to 60 °C at 1 °C /cycle. This process was named PCR4.

Third, the product of PCR4 was treated as a DNA template. PCR5 was performed using primer pair J (Table 3). Namely, the PCR mixture contained the product of PCR4 (50–100 ng), 0.1 μ M of the specific primers (Table 3), 80 μ M of deoxynucleotide triphosphates (dNTPs), 0.5 U LA *Taq* polymerase, and 10 μ l of 2 × GC Buffer II (Takara Bio). PCR was performed using the following conditions: 95 °C for 10 min; the first thermocycling consisting of 8 cycles at 95 °C for 30 s, decreasing the annealing temperature from 68 °C to 60 °C at 1 °C /cycle for 30 s, and 72 °C for 3 min; the second thermocycling consisted of 25 cycles at 95 °C for 30 s, 60 °C for 15 s, and 72 °C for 3 min. Then a final extension was performed at 72 °C for 10 min. The products (Up + *Tmp* + Down, eUTD) of PCR5 were analysed using a 1% agarose gel.

4.10 | Construction of the truncated homologous recombination fragments

The truncated homologous recombination fragments were constructed by touchdown PCR. The Up + truncated fragment (eUT') and truncated + Down fragment (eT'D) were amplified using the primers (Table 3; K, L) and the DNA template PMD-19-UTD. The PCR system was similar to PCR5 mentioned before. The annealing temperatures are shown in Table 3.

4.11 | Design and construction of the knockout plasmid pUT-eUTD

The knockout plasmid pUT-UTD derived from pUTTns (Li et al., 2009) was constructed to edit the endo-1,4- β -glucanase gene (*BpEG*, accession number: MH733823) in *B. pyrrocinia* JK-SH007. The *Tmp* gene with the promoter was obtained from the plasmid pHKT2 (Tomlin et al., 2004) by PCR amplification using the Tmp-F/Tmp-R primer pair (Table 3). The *Tmp* gene was cloned into the pMD19-T vector (Takara Bio).

Owing to the limitation of restriction sites, we used homologous cloning to construct the knockout plasmid. The fragment derived from pUTTns was obtained using reverse PCR (with the simple1/ simple2 primers), which involved *Amp*, *mob* RP4, R6K π ori, and the *lac* operator. Namely, the elements of the Tn5 IE end, *tnpA*, *sacB*, *Kan*, similar to *sacB*, MCS, and Tn5 OE end were removed. The three-fragment (Up + *Tmp* + Down) product amplified using the SP-EG-F/SP-EG-R primer pair, with a partial homologous sequence of the plasmid, was inserted into the fragment derived from pUTTns using recombinase (ClonExpress II One Step Cloning Kit), as described by the manufacturer (Vazyme Biotech). The newly constructed recombinant plasmid had the element of the R6K π origin, so *E. coli* DH5 $\alpha_{\lambda pir}$ was used as a host strain for cloning the plasmid, as described by Li et al. (2009). The resulting vector was designated pUT-eUTD.

4.12 | Effects of different DNA forms on gene knockout

The following four different DNA forms to achieve HR were explored by the optimized transformation method mentioned earlier: HR (eUTD) produced by PCR5, truncated HR fragments (eUT' and eT'D), the plasmid PMD-19-eUTD, and the plasmid pUT-eUTD.

4.13 | Detection of the knockout gene by PCR

Colony PCR detection of the GC-rich gene was present. The amplification system used 20 μ l: 10 μ l of 2 × Taq FroggaMix (FroggaBio), 1 μ l of 10 μ M forward and reverse primers, respectively (Table 3), 1 μ l of *B. pyrrocinia* JK-SH007, 2 μ l of dimethyl sulphoxide (DMSO), and 6 μ l of double distilled water to fill the reaction volume up to 20 μ l. The PCR was performed using the following conditions: 95 °C for 10 min, thermocycling consisting of 33 cycles at 95 °C for 30 s, annealing temperature (Table 3) for 30 s, and 72 °C for 1 kb/min, with a final extension at 72 °C for 10 min.

4.14 | Southern blotting

The Southern blotting was performed using Digoxigenin (DIG) High Prime DNA Labeling and Detection Starter Kit I, as described by the manufacturer (Roche Applied Science). The *Tmp* gene was obtained by PCR and purified using High Pure PCR Product Purification Kit (Roche Applied Science). The purified fragment of *Tmp* was labelled using DIG-High Prime solution from the kit.

4.15 | Congo red plate test and reducing sugar release test

Both wild-type and mutant *B. pyrrocinia* JK-SH007 strains were dropped on the CMC agar plate containing 0.7% agar (Chen, Ye, Susta Kameshwar et al., 2020). All plates were incubated at 28 °C for 5 days and stained with 0.1% (wt/vol) Congo red solution for 30 min and visualized by washing with 1 M sodium chloride solution for 10 min. The hydrolysis ability was calculated according to the method of Guo et al. (2017).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

F.C., Q.W., and J.Y. conceived and designed the project. F.C., W.L., and C.C. performed the experiments. F.C. and W.L., analysed the data. F.C., C.C., W.W., Q.W., and J.Y wrote the manuscript. All the authors discussed the results and approved the final version.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in the manuscript and supporting files.

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14

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Molecular Plant Pathology

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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