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An efficient system for the generation of marked genetic mutants in members of the genus *Burkholderia*

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Abbreviations

BHR, broad host-range

MCS, multiple cloning site

TIR, translation initiation region

Cm^R, chloramphenicol resistance/resistant

Cm^S, chloramphenicol sensitive

Km^R, kanamycin resistance

Tc^R, tetracycline resistance

Tp^R, trimethoprim resistance

29 **Highlights**

30 • We have constructed improved antibiotic-resistance cassettes and suicide vectors for
31 use in members of the *Burkholderia cepacia* complex (Bcc) and related species.

32 • These vectors facilitate construction of mutants by gene disruption with antibiotic-
33 resistance markers.

34 • We have validated the utility of the vectors for marked genetic inactivation in *B.*
35 *cenocepacia*, *B. lata* and *B. thailandensis*.

36

37 **Keywords**

38 Marked mutation; gene inactivation; *Burkholderia cepacia* complex; suicide vector; antibiotic-
39 resistance.

40

41 # Abstract

42 To elucidate the function of a gene in bacteria it is vital that targeted gene
43 inactivation (allelic replacement) can be achieved. Allelic replacement is often carried out by
44 disruption of the gene of interest by insertion of an antibiotic-resistance marker followed by
45 subsequent transfer of the mutant allele to the genome of the host organism in place of the
46 wild-type gene. However, due to their intrinsic resistance to many antibiotics only selected
47 antibiotic-resistance markers can be used in members of the genus *Burkholderia*, including
48 the *Burkholderia cepacia* complex (Bcc). Here we describe the construction of improved
49 antibiotic-resistance cassettes that specify resistance to kanamycin, chloramphenicol or
50 trimethoprim effectively in the Bcc and related species. These were then used in
51 combination with and/or to construct a series enhanced suicide vectors, pSHAFT2,
52 pSHAFT3 and pSHAFT-GFP to facilitate effective allelic replacement in the Bcc. Validation
53 of these improved suicide vectors was demonstrated by the genetic inactivation of selected
54 genes in the Bcc species *Burkholderia cenocepacia* and *B. lata*, and in the non-Bcc species,
55 *B. thailandensis*.

56

57 # 1. Introduction

58 *Burkholderia cepacia* complex (Bcc) are a group of at least twenty closely related
59 Gram-negative bacterial species of the *Burkholderia* genus, which are phenotypically similar
60 but genetically different (Vandamme et al., 1997; De Smet et al., 2015). These species are
61 found ubiquitously in the environment, including soil, water and the rhizosphere of some
62 plants (Coenye and Vandamme, 2003). Many Bcc species have also been isolated from the
63 sputum of cystic-fibrosis (CF) patients, identifying them as opportunistic pathogens of those
64 with lung disease and weakened immune systems (Henry et al., 1997; Speert et al., 2002).
65 Colonisation of the lungs by Bcc can result in several clinical outcomes, including chronic-
66 infection with gradual lung deterioration and a rare adverse reaction known as Cepacia
67 syndrome, which leads to rapid lung decline, septicaemia, necrotizing pneumonia and is
68 often fatal (Isles et al., 1984). The most prevalent causative agents of Bcc infections in CF-
69 patients in the UK, Canada and USA are *B. cenocepacia* and *B. multivorans* (Drevinek and
70 Mahenthiralingam, 2010; De Soyza et al., 2010; Zlosnik et al., 2015). Unfortunately, Bcc
71 infections can be difficult to treat due to the high intrinsic resistance of these bacteria to
72 many antibiotics. Over recent years restrictions have been placed on individuals colonized
73 with Bcc to prevent transmission of Bcc infections, by limiting their contact with at risk
74 members of the population (Ledson et al., 1998).

75 Efforts have been made to understand the virulence mechanisms used by Bcc
76 bacteria that allow them to be successful pathogens. These include mechanisms of bacterial
77 cell invasion, intracellular survival, quorum sensing, iron acquisition, and protein secretion
78 (Martin and Mohr, 2000; Sokol et al., 2003; Visser et al., 2004; Mullen et al., 2007; Aubert et
79 al., 2008; Uehlinger et al., 2009; Somvanshi et al., 2010). A key factor to aid in the
80 characterization of these traits is the ability to manipulate the genome of Bcc species.
81 Methods for targeted gene inactivation in *Burkholderia* have largely relied on the disruption
82 of the target chromosomal gene with an antibiotic-resistance marker, either through
83 employing an integrative vector (Flannagan et al., 2007; Chapalain et al., 2013) or by allelic
84 replacement whereby the wild type gene is replaced by a copy of the target gene that is
85 inactivated with an antibiotic-resistance cassette (Huber et al., 2001; Malott et al., 2005;

86 Agnoli et al., 2006). In the latter, two recombination crossover events are selected for (one
87 occurring either side of the lesion in the target gene) and no vector sequences remain on the
88 chromosome in the mutant. Vectors for generation of such marked mutants include plasmids
89 in the pEX18 and pEX19 series (Hoang et al., 1998), and a vector constructed in our lab,
90 pSHAFT (Agnoli et al. 2006).

91 Due to their high level of intrinsic resistance to many antibiotics, the number of
92 commonly employed resistance markers that can be used for genetic manipulation of Bcc
93 species is generally restricted to those specifying increased resistance to trimethoprim,
94 chloramphenicol and tetracycline. All three markers can be selected for in single copy in *B.*
95 *cenocepacia* (Farmer and Thomas, 2004; Asghar et al., 2011; Ryan et al., 2013). For
96 aminoglycoside-sensitive strains, whether naturally occurring (such as *B. cenocepacia* strain
97 H111) or engineered, the *aac* and *aph* markers, which specify resistance to the
98 aminoglycosides gentamycin and kanamycin, respectively, are selectable in single copy and
99 can therefore also be used in allelic replacement experiments (Huber et al., 2001; Hamad et
100 al., 2010; Inhülsen et al., 2012; Carlier et al., 2014).

101 Allelic replacement in bacteria involving disruption of a target gene with a selectable
102 resistance marker can be problematic if the host bacterium already exhibits a high degree of
103 intrinsic resistance to the antibiotic, thereby precluding selection of the marker. This can be
104 ameliorated if the antibiotic-resistance gene is efficiently expressed. However, the use of
105 strong promoters to drive high-level transcription of the marker gene can exert detrimental
106 polar effects that include destabilisation of plasmids housing such markers or unwanted or
107 toxic expression of downstream genes following insertion of the marker in the host genome
108 (Stueber and Bujard, 1982; Stassi and Lacks, 1982; Schrecke et al., 2013; our published
109 observations). In these situations the negative effects of very active promoters can be
110 circumvented by placement of a strong transcription terminator downstream of the selectable
111 marker gene (Gentz et al., 1981; Fellay et al., 1987). Here we describe the construction of
112 novel antibiotic-resistance cassettes where we have employed these principles either to
113 increase expression of the antibiotic-resistance marker to facilitate its selection in a single
114 copy or to prevent detrimental polar effects by blocking transcriptional readthrough into

115 downstream genes. These cassettes were used to build improved suicide vectors derived
116 from the vector pSHAFT and were also used in conjunction with these vectors for allelic
117 replacement in the Bcc and other members of the genus *Burkholderia*.
118

119 2. Materials and methods

120 2.1. Strains, plasmids, and growth conditions

121 The bacterial strains and plasmids used in this study are given in supplementary
122 Table S1. For cultivation of bacteria, strains were routinely grown in/on LB medium at 37°C.
123 For selection of trimethoprim resistance in *E. coli* iso-sensitest agar (Oxoid) was employed,
124 whereas for *B. cenocepacia* M9-minimal salts agar containing 0.5% glucose was used
125 (except where indicated). M9-minimal salts used here contained 42 mM Na_2HPO_4, 22 mM
126 KH_2PO_4, 19 mM NH_4Cl, 9 mM NaCl, 1 mM $MgSO_4$ and 100 μM $CaCl_2$. For selection of
127 kanamycin resistance in *B. cenocepacia* Lennox agar was utilised. Antibiotics were used,
128 when appropriate, at the following concentrations for selection of plasmids and antibiotic-
129 resistance cassettes in *E. coli* and *Burkholderia* species, as indicated: ampicillin, 100 μg/mL
130 (*E. coli*); kanamycin, 50 μg/mL (*E. coli* and *B. cenocepacia*); chloramphenicol, 25 μg/mL (*E.*
131 *coli*) and 50 μg/mL (*B. cenocepacia*); trimethoprim, 25 μg/mL (*E. coli*, *B. cenocepacia* and *B.*
132 *lata*) and 50 μg/mL (*B. thailandensis*). For *B. cenocepacia* strains exhibiting a high intrinsic
133 resistance to trimethoprim, such as K56-2, this antibiotic was included in the medium at 100
134 μg/mL.
135

136 2.2. DNA preparation and manipulation

137 Recombinant DNA techniques were performed essentially as described in Sambrook
138 et al. (1989). DNA amplification by PCR was performed as standard according to the
139 manufacturer's instructions using KOD DNA polymerase enzyme (Millipore) and a G-storm
140 thermocycler. Primers used in this study are indicated in supplementary Table S2, and were
141 purchased from Eurogentec, Belgium. To extract DNA for PCR, bacterial colonies were
142 resuspended in 200 μL TE buffer (10 mM Tris, 1.0 mM EDTA (pH 8.0)), boiled for 7 minutes,
143 and then centrifuged to pellet cell debris. The supernatant was retained for use in PCR

144 (referred to as ‘boiled lysate’). PCR products were purified from solution or by agarose gel-
145 extraction using a QIAquick PCR purification kit (Qiagen). DNA restriction enzymes were
146 purchased from Promega or New England Biolabs. DNA was ligated using T4 DNA ligase
147 (Promega). 5’ DNA overhangs were filled-in using DNA I polymerase Klenow fragment
148 (Promega). Sequencing was performed by the Core Genomic Facility at The University of
149 Sheffield, UK.
150
151 **2.3. Plasmid constructions**
152 Plasmids were extracted from saturated bacterial cultures using a spin-column-based
153 plasmid mini-prep extraction kit (Thermo Scientific). Plasmids were transferred into *E. coli* by
154 heat-shock assisted transformation according to Hanahan (1983).
155 To construct p34E-Km, the Tn*5*-derived *aphA2* gene, including its promoter
156 (Rothstein and Reznikoff, 1981), was excised from pKNOCK-Km as a *Mlu*I fragment, end-
157 filled with DNA polymerase I Klenow fragment and ligated between the filled *Eco*RI sites of
158 p34E-Tp.
159 To generate p34E-Cm2, the *catA2* gene of the plasmid pSHAFT was amplified with
160 primers p34E-Cmfor2 and p34E-Cmrev, which incorporated a synthetic promoter (-10 and -
161 35 elements) upstream of the *catA2* TIR in the amplified product. The 823 bp amplicon was
162 restricted with *Eco*RI and used to replace the *Eco*RI-excised Km^R cassette of p34E-Km.
163 To construct p34E-TpTer, first a 306 bp DNA fragment containing the *rrnB* T1T2
164 terminators was amplified from the plasmid pEA302T with rrnBterfor and rrnBterrev and
165 restricted with *Bam*HI and *Hin*dIII, following which it was ligated between the corresponding
166 restriction sites of pUC18 to give pUC18Ter. Second, the megaprimer variation of SOE PCR
167 (Perrin and Gilliland, 1990) was used to fuse the *rrnB* T1T2 terminators to the 3’ end of
168 *dfrB2*. To do this, the *dfrB2* gene (and native promoter) was amplified from p34E-Tp with
169 primer Tp(forward), and the fusion primer Tp(reverse) that was complementary to the 3’ end
170 of the *dfrB2* gene, including the stop codon, and contained a 5’ tail with 19 bp of homology to
171 the upstream region of the *rrnB* T1T2 DNA fragment. The resultant amplicon served as a
172 forward megaprimer in combination with reverse primer rrn(R), to amplify a 903 bp *dfrB2*-

173 *rrnB* T1BT2 fusion fragment from pUC18Ter. This product was then ligated between the
174 *Eco*RI and *Hin*dIII sites of pUC19, giving rise to pUC19TpTer. Last, the TpTer cassette of
175 pUC19TpTer was excised as a *Stu*I fragment and ligated between the filled-in *Eco*RI sites of
176 p34E-Km, thereby replacing the Km^R cassette.

177 To construct pSHAFT2, the *catA2* region of pSHAFT was amplified with a pair of
178 primers, pSHOOTERfor2 and pSHOOTERrev. The 871 bp product was digested with *Bam*HI
179 and *Sal*I and ligated between the *Bgl*II and *Sal*I sites of pSHAFT, thereby replacing the Ω-
180 Cm interposon.

181 To construct pSHAFT3, first, a double-stranded oligonucleotide was generated by
182 annealing oligonucleotides pSHAFT3MCSfor and pSHAFT3MCSrev. This was achieved by
183 combining 45 μM of each oligonucleotide in 1x KOD DNA polymerase reaction buffer
184 (Millipore) and 1 mM $MgCl_2$, incubating at 90°C for 10 minutes followed by incubation at
185 room temperature for 1 hour and subsequent purification of the double-stranded
186 oligonucleotide. pSHAFT2 was restricted with *Not*I and *Eco*RI and ligated to the double-
187 stranded oligonucleotide, to generate pSHAFT3.

188 To construct pSHAFT-GFP, an 866 bp DNA fragment containing the *gfp* coding
189 sequence and Shine-Dalgarno was amplified from pBHR1-GFP with primers GFPfor and
190 GFPrev, purified and digested with *Bam*HI and *Sal*I. This was then ligated between *Bgl*II-*Sal*I
191 sites of pSHAFT to replace the 3.7 kb Ω-Cm fragment of this vector.

192 The sequences of p34E-Km, p34E-Cm2, p34E-TpTer, pSHAFT, pSHAFT2,
193 pSHAFT3 and pSHAFT-GFP were deposited in GenBank database, and given the
194 accession numbers KX485327, KX485328, KX485329, KX485330, KX485332, KX485333
195 and KX485331, respectively. As we observed that the nucleotide sequence of the *dfrB2*
196 cassette in p34E-Tp as deposited in the database is incorrect, we have also submitted an
197 amended sequence of this plasmid that has been assigned accession number KX485326.
198 pUC18Ter and pUC19TpTer were assigned accession numbers KX527623 and KX527624,
199 respectively.

200

201 **2.4. Gene replacement in *B. cenocepacia***

202 To inactivate chromosomal genes in *Burkholderia* using pSHAFT2 or pSHAFT3, the
203 antibiotic counter-selection-based strategy used for allelic replacement by pSHAFT was
204 employed (Agnoli et al. 2006). For isolation of a *B. cenocepacia* BCAM0195::Tp mutant, a
205 3.64 kb DNA fragment containing the BCAM0195 gene orthologue in strain H111 was
206 amplified from a boiled lysate using primers BCAM0195for and BCAM0195rev, restricted
207 with *Hin*dIII and *Bam*HI and ligated between the corresponding sites of pBBR1MCS-1,
208 generating pBBR1-BCAM0195'. The cloned 3.11 kb gene fragment was then disrupted by
209 ligation of the p34E-Tp-derived Tp^R cassette as a *Sma*I-fragment between two *Pml*I sites
210 located 1.58 kb apart within BCAM0195, generating pBBR1-ΔBCAM0195'::Tp. The 2.04 kb
211 ΔBCAM0195'::Tp allele was transferred to pSHAFT2 as a *Xho*I-*Xba*I fragment to give
212 pSHAFT2-ΔBCAM0195'::Tp. This plasmid was conjugated into *B. cenocepacia* H111 using
213 *E. coli* donor strain S17-1(λpir) according to Herrero et al., (1990) and de Lorenzo and
214 Timmis (1994), and *B. cenocepacia* exconjugants were selected on M9-glucose agar
215 containing trimethoprim. Exconjugants were patched onto the same medium and also LB
216 agar containing chloramphenicol. Exconjugants that were chloramphenicol-sensitive were
217 identified as candidate ΔBCAM0195::Tp mutants. Due to the loss of short regions of DNA at
218 the 5' and 3' ends of BCAM0195 during the construction of pSHAFT2-ΔBCAM0195'::Tp,
219 potential ΔBCAM0195::Tp mutants could be verified by PCR using the original
220 BCAM0195for and BCAM0195rev primers, as they annealed to genomic sequences located
221 a short distance either side of the region of DNA that was also present in pSHAFT2-
222 ΔBCAM0195'::Tp.

223 For isolation of a BCAL1709::TpTer mutant in *B. cenocepacia* AHA27, a 1.354 kb
224 DNA fragment containing the 3' region of the BCAL1709 orthologue was amplified from *B.*
225 *cenocepacia* Pc715j genomic DNA using primers BCAL1709for and BCAL1709rev,
226 restricted with *Xba*I *and Xho*I, and the resulting 1.297 kb amplicon was ligated between the
227 corresponding sites of pSHAFT-GFP to give rise to pSHAFT-GFP-BCAL1709. The
228 BCAL1709 gene was then disrupted by ligation of the p34E-TpTer-derived Tp^R cassette as a

229 *Sma*I fragment into a unique *Zra*I site within BCAL1709, resulting in pSHAFT-GFP-
230 BCAL1709::TpTer. This plasmid was then conjugated into AHA27 and exconjugants
231 containing the BCAL1709::TpTer allele within the genome were selected for as described for
232 construction of the BCAM0195::Tp mutant. The presence of *gfp* on pSHAFT-GFP causes
233 recipient colonies to fluoresce under UV light, and was used to distinguish between
234 recombinants that arose through a single crossover (i.e. plasmid integration - fluorescent)
235 and a double crossover (i.e. allelic replacement - non-fluorescent). 50 trimethoprim-resistant
236 exconjugants were patched on duplicate IST agar plates containing trimethoprim. One of
237 each pair of plates was exposed to UV light on a transilluminator in the dark to identify non-
238 fluorescent candidate AHA27 BCA1709::TpTer mutants. Candidate mutants from the non-
239 irradiated duplicate plate(s) were verified by PCR using primers BCAL1709forOut and
240 BCAL1709revOut, which annealed to genomic sequences a short distance either side of the
241 region of DNA that was also present in pSHAFT-GFP-BCAL1709::TpTer.
242

243 # 3. Results and discussion

244 ## 3.1. Construction of antibiotic resistance cassettes for genetic manipulation of the
245 ## *Burkholderia cepacia* complex

246 To facilitate the generation of mutants in *Burkholderia* through insertional inactivation
247 of chromosomal target genes by antibiotic resistance markers and the construction of useful
248 plasmid vectors that would allow an investigation into gene function and control in members
249 of this genus, we made derivatives of the cassette cloning vector, p34E (Tsang et al., 1991),
250 harbouring the kanamycin- (*aphA2*), chloramphenicol- (*catA2*) and trimethoprim- (*dfrB2*),
251 resistance genes.

252 First, p34E-Km was constructed by replacing the trimethoprim-resistance (Tp^R)
253 cassette of p34E-Tp with the Tn*5*-derived *aphA2* (*aph(3')-II*) gene from pKNOCK-Km (Figure
254 1). Although a similar kanamycin-resistance cassette (Km^R) plasmid, p34S-Km (which
255 contains the Tn*903*-derived *aph(3')-Ia* gene), has been previously described (Dennis and
256 Zylstra, 1998a), the Tn*5*-derived antibiotic resistance marker in p34E-Km offers the
257 advantage that it makes available the *Hin*dIII and *Sma*I sites flanking the cassette (as they

258 do not cut within the *aphA2* gene) and also contains flanking *Eco*RI sites. Both types of
259 cassette are of a similar size (~1.3 kb). A shorter variant of the Tn903-based Km^{R} cassette
260 lacking the internal *Hin*dIII and *Sma*I sites was subsequently constructed, but it lacks the
261 flanking *Eco*RI sites and the possibility for directional cloning associated with p34E-Km
262 (Dennis and Zylstra, 1998b).

263 Most plasmids used for genetic manipulation that specify chloramphenicol resistance
264 (Cm^{R}) harbour the Tn*9*-derived *catA1* gene (or one that is closely related) that specifies an
265 enzyme belonging to group 1 of the type A chloramphenicol acetyltransferases (CATs)
266 (Shaw, 1983; Schwarz et al., 2004). A notable exception is mini-Tn*5*Cm, which contains the
267 *catA2* gene (de Lorenzo et al., 1990). Pertinently, chloramphenicol resistance conferred by
268 this mini-transposon is selectable in single copy in *B. cenocepacia* (Farmer and Thomas,
269 2004). However, the *catA2* gene specifying this resistance is located on a large DNA
270 fragment (the ~3.7 kb Ω-Cm interposon) that was used to assemble the transposon and is
271 not available as a small cassette (Fellay et al., 1987; de Lorenzo et al., 1990). The Ω-Cm
272 interposon includes a 2.8 kb DNA fragment derived from the cloning vector pKT210 which
273 contains the 214 codon *catA2* ORF and several other predicted ORFs found on the naturally
274 occurring BHR plasmid pSa (Bagdasarian et al., 1981; Tait et al., 1982; Shaw, 1983; Fellay
275 et al., 1987). In order to generate a compact Cm^{R} cassette containing an efficiently
276 expressed *cat* gene, the *catA2* gene contained on pSHAFT (a suicide plasmid derived from
277 pUTmini-Tn*5*Cm (see below)), was modified to incorporate a synthetic σ^{70}-dependent
278 promoter upstream of the *catA2* TIR. The modified *catA2* gene was used to replace the Km^{R}
279 cassette of p34E-Km, to generate p34E-Cm2 (Figure 1). The Cm^{R} cassette in p34E-Cm2,
280 can be transferred to other vectors using any of the flanking restriction sites except *Sma*I
281 (Figure 1), although even here, selection for chloramphenicol resistance would permit
282 selection for the products of a tripartite ligation between the target vector and the two *Sma*I-
283 generated cassette fragments. As an alternative, either of the blunt end-generating *Sac*I
284 isoschizomers, *Eco*53kI or *Ecl*136II, may be used.

285 The most similar vectors available as sources of a Cm^{R} cassette are p34S-Cm and -
286 Cm2 (Dennis and Zylstra, 1998a; 1998b). Rather than the *catA2* gene present in p34E-Cm2,

287 these other plasmids contain the Tn*9*-derived *catA1* gene and the cassettes are slightly
288 longer (918 bp rather than 823 bp). p34S-Cm contains an internal *Eco*RI site whereas in
289 p34S-Cm2 this site has been removed. However, unlike p34E-Cm2, p34S-Cm2 lacks
290 flanking *Eco*RI sites.

291 The trimethoprim-resistance gene (*dfrB2*) present on the cassette vectors p34E-Tp
292 and p34S-Tp is under control of the very strong PcS integron promoter (also known as P1)
293 (Lévesque et al., 1994; DeShazer and Woods, 1996; Dennis and Zylstra, 1998a; Jové et al.,
294 2010). We have observed that insertion of the Tp^R cassette in certain orientations in some
295 plasmid vectors is not possible (see below) and when used in allelic replacement
296 experiments it can result in impaired growth of the resultant mutants, presumably due to
297 overexpression of chromosomal genes located downstream of the integrated cassette (S.S.
298 and M.S.T., unpublished observations). To circumvent this problem, we modified the Tp^R
299 cassette by placing the efficient T1T2 transcription terminators from the *E. coli rrnB*
300 ribosomal RNA operon, downstream of the *dfrB2* coding sequence (Brosius, 1984; Orosz et
301 al., 1991). To do this, we started with pEA302T, a plasmid into which a 500 bp *Eco*RI
302 fragment containing the 5S rRNA gene and the T1T2 terminators was cloned in order to
303 prevent readthrough transcription of the *tac* promoter into the replication region (Amann et
304 al., 1983). A DNA fragment containing only the *rrnB* T1T2 terminators was amplified using
305 primers that also incorporated additional restriction sites at the flanking regions of the
306 fragment, and was subsequently transferred to pUC18 to give pUC18Ter.

307 The *rrnB* terminators (from pUC18Ter) were then fused to the *dfrB2* gene (from
308 p34E-Tp) by the megaprimer variation of the SOE PCR technique (Perrin and Gilliland,
309 1990), and the amplicon was ligated into pUC19, giving rise to pUC19TpTer. Although the
310 TpTer DNA fragment in pUC19TpTer can be transferred as a *Stu*I or *Nde*I cassette, to
311 increase its versatility, it was transferred into p34E-Km, substituting for the Km^R cassette.
312 The resultant plasmid, p34E-TpTer, is analogous to p34E-Tp but has strong transcription
313 termination signals located downstream of the *dfrB2* gene and includes the addition of *Nde*I
314 sites in the flanking MCSs (Figure 1).

315

316 **3.2. Construction of allelic replacement vectors, pSHAFT2 and pSHAFT3, for**
317 **generation of marked mutants in the *Burkholderia cepacia* complex**

318 We sought to improve upon existing vectors used for allelic replacement in the Bcc. A
319 useful vector for insertional inactivation of chromosomal genes with selective markers
320 (usually antibiotic resistance cassettes) is pSHAFT, an R6K-based suicide plasmid (Figure
321 2) (Agnoli et al., 2006). This vector was derived from the transposon delivery plasmid,
322 pUTmini-Tn*5*Cm (de Lorenzo et al., 1990), by deletion of the *tnp* (transposase) gene and
323 one of the 19 bp repeat sequences (the ‘I end’) that flank mini-Tn*5*Cm, and therefore it
324 cannot be mobilised in the presence of transposase provided in *trans*. pSHAFT is also
325 devoid of the *pir* gene, which specifies the plasmid replication initiator protein, π, and can
326 therefore only replicate in bacteria containing the *pir* gene, such as *E. coli* CC118(λpir)
327 (Herrero et al., 1990; Rakowski and Filutowicz, 2013). Along with the chloramphenicol-
328 resistance marker carried by mini-Tn*5*Cm, pSHAFT also contains the origin of transfer (*oriT*)
329 from RP4 (RK2) that allows for efficient conjugal transfer of the plasmid to a variety of Gram-
330 negative bacterial species. Following transfer of a Bcc gene (or gene fragment) to pSHAFT,
331 the gene is disrupted by insertion an antibiotic-resistance cassette (other than Cm^R) that is
332 selectable in the Bcc. The plasmid is then introduced into a Bcc strain and selection for the
333 presence of the antibiotic-resistance cassette (Ab^R) is applied. As the plasmid cannot
334 replicate in Bcc, only recombinants are obtained in which the plasmid has recombined with
335 the host genome at the locus that is homologous to the Bcc DNA present on the plasmid.
336 This may result in integration of the plasmid into the genome (single crossover, Cm^R Ab^R) or
337 allelic replacement (double crossover, Cm^S Ab^R).

338 However, the major drawback with pSHAFT is the limited number of unique
339 restriction sites that can be used for inserting mutant alleles for subsequent chromosomal
340 targeting. One reason for this is the duplication of restriction sites either side of the Cm^R
341 element during the assembly of mini-Tn*5*Cm in the progenitor plasmid pUT (de Lorenzo and
342 Timmis, 1994). Moreover, as discussed above, the cassette specifying resistance to
343 chloramphenicol is unnecessarily large, as mini-Tn*5*Cm was originally constructed by

344 inserting the ~3.7 kb Ω-Cm interposon between the 19 bp I and O ends of Tn*5* carried by
345 pUT (de Lorenzo et al., 1990).

346 To improve the utility of pSHAFT, the vector was modified by replacing the
347 interposon with a much shorter DNA fragment containing the *catA2* gene under control of a
348 synthetic σ^{70}-dependent promoter as also incorporated in p34E-Cm2, thereby decreasing
349 the plasmid size from 7.5 to 4.6 kb. The new plasmid, pSHAFT2, contains 11 unique
350 restriction sites located downstream of the *catA2* gene, one of which (*Stu*I) allows the
351 cloning of blunt-ended fragments, while the *Bgl*II site can also accommodate *Bam*HI-
352 generated fragments. At the *catA2*-distal end of the MCS are three closely spaced *Eco*RI
353 sites that can be considered as an additional single unique site for cloning purposes (Figure
354 2).

355 The versatility of pSHAFT2 was then improved by replacing the region extending
356 from the *Not*I site in the MCS to the most distant of the three *Eco*RI sites by a double-
357 stranded oligonucleotide that contained internal *Spe*I and *Apa*I sites. This also resulted in a
358 net loss of two of the three *Eco*RI sites present in pSHAFT2 and removal of the mini-Tn*5* O
359 end that originated from the progenitor plasmid of pSHAFT. The new plasmid, pSHAFT3
360 (4.5 kb), is shown in Figure 2.

361

362 3.3. Construction of an allelic replacement vector, pSHAFT-GFP, that allows 363 fluorogenic detection of integration events in the *Burkholderia cepacia* complex 364 during generation of marked mutants

365 To allow for inactivation of chromosomal genes where the *Burkholderia* strain already
366 harbours a chloramphenicol resistance marker or to disrupt chromosomal genes with a Cm^R
367 cassette we constructed pSHAFT-GFP (Figure 2). This plasmid is analogous to pSHAFT2
368 but with the *catA2* gene replaced by the *gfp* gene, which therefore allows for fluorogenic
369 detection of recombinants containing the genomically integrated vector. The forward primer
370 used to amplify the *gfp* gene specified recognition sites for the restriction enzymes *Sal*I,
371 *Sma*I, *Bgl*II, *Xba*I, *Kpn*I, *Xho*I and *Stu*I and an artificial promoter containing the consensus -
372 35 and -10 elements for σ^{70}-dependent promoters, whereas the reverse primer specified

373 only a *Bam*HI site. This vector is used in the same way as pSHAFT2 except for the fact that
374 single and double crossover recombinants harbouring the selectable marker used to
375 inactivate the target chromosomal gene are distinguished from each other by screening
376 colonies for the absence of yellow-green fluorescence. Although this usually requires
377 exposure to UV in the dark (a UV transilluminator works well in this regard), in some
378 mutagenesis experiments the presence of the GFP marker can be discerned without
379 recourse to UV exposure by the yellow-green colour of the colonies.
380

381 **3.4. Isolation of marked mutants in *B. cenocepacia* using the pSHAFT-vector series**

382 To inactivate chromosomal genes in *Burkholderia* using the pSHAFT-vector series,
383 DNA fragments of ≥ 1.0 kb containing the target gene (or gene fragment) are inserted into
384 the MCS and then subsequently disrupted by insertion of an antibiotic resistance cassette
385 (usually Tp^R, Km^R or Tc^R) such that at least 0.5 kb of homology occurs between the cloned
386 DNA target region and the chromosome either side of the lesion (Figure 3A). Depending on
387 the availability of restriction sites, in some cases we have found it more convenient to first
388 clone the target DNA sequence into a general-purpose plasmid vector and then introduce
389 the antibiotic resistance cassette before transferring the disrupted DNA fragment to the
390 suicide vector. In using these plasmids to inactivate chromosomal genes in *Burkholderia*, we
391 have observed that the Tp^R cassette can often only be inserted into target genes in one
392 orientation, such that transcription is directed towards the *catA2* gene, and away from the
393 origin of replication. We assume this is due to transcriptional destabilisation of plasmid
394 replication functions (Gentz et al., 1981; Stueber and Bujard, 1982; Stassi and Lacks, 1982)
395 as this problem does not occur with the TpTer cassette (our unpublished observations).

396 Once the disrupted gene or gene fragment has been introduced into pSHAFT2, it is
397 transferred to *Burkholderia* and selection is applied for the antibiotic resistance marker that
398 was used to disrupt the gene or gene fragment present in the suicide vector, thus identifying
399 strains that have integrated the disrupted allele into the genome by homologous
400 recombination. Recombinants are of two types: those that are the result of a single
401 crossover, in which the entire plasmid has integrated into the genome at the target gene

402 locus, and those that are the result of a double crossover in which only the mutant allele has
403 been transferred to the genome in place of the original wild type gene (Figure 3A). In the
404 former, wild type and mutated copies of the gene (or gene fragment) are present in the
405 genome of the recipient bacterium, which will also specify increased resistance to
406 chloramphenicol due to the integrated vector. These are identified by 'patching' out
407 recombinants on LB agar containing 50 μg/ml chloramphenicol. Chloramphenicol-sensitive
408 recombinants are screened for the presence of the mutated allele (and absence of the wild
409 type allele) by PCR using primers that anneal to genomic sequences flanking the region that
410 was originally cloned in the suicide vector.

411 We have successfully used pSHAFT2 to generate several marked mutants within
412 Bcc species. This included the insertional inactivation of the I35_0520 gene in *B.*
413 *cenocepacia* strain H111 (the orthologue of BCAM0195 in strain J2315 and will be referred
414 to as such hereon), which was disrupted by the Tp^R cassette derived from p34E-Tp.
415 BCAM0195 is predicted to encode a non-ribosomal peptide synthetase (NRPS) of unknown
416 function. During the isolation of the ΔBCAM0195::Tp mutant, we observed that out of 50
417 trimethoprim resistant exconjugants, 16 were chloramphenicol sensitive. Three of these
418 were verified as ΔBCAM0195::Tp mutants by PCR, where the DNA fragment amplified from
419 BCAM0195 in the mutants was ~900 bp smaller than the wild-type, due to replacement of a
420 large segment of BCAM0195 by the Tp^R cassette in the ΔBCAM0195::Tp allele (Figure 3B).
421 We have also used this plasmid to introduce an *orbI*::Tp allele into the genome of *B. lata*
422 using the same selection conditions employed for allelic replacement in *B. cenocepacia*
423 (results not shown). The applicability of the pSHAFT vectors to other non-Bcc species within
424 the genus was established by generating a type VI secretion system mutant of *B.*
425 *thailandensis* (a member of the 'pseudomallei' group) in an analogous fashion using
426 pSHAFT3 as the vector and the TpTer cassette to disrupt the *tssK* gene (results not shown).

427 To demonstrate the utility of pSHAFT-GFP it was used to inactivate the orthologue of
428 the *B. cenocepacia* J2315 BCAL1709 gene that is present in the siderophore-negative *B.*
429 *cenocepacia* mutant AHA27. AHA27 is a derivative of *B. cenocepacia* strain Pc715j that
430 contains a mini-Tn*5*CmlacZYA insertion in the *pobA* gene encoding a phosphopantetheinyl

431 transferase (PPTase) required for activating NRPS enzymes, and thereby specifies higher
432 levels of resistance to chloramphenicol than the parent strain (Asghar et al., 2011). Based
433 on amino acid sequence alignment, BCAL1709 is very likely to encode a putative TonB-
434 dependent receptor thought to be involved in the uptake of an unknown xenosiderophore
435 complexed with ferric iron (M.S.T., unpublished results). Following cloning of a BCAL1709
436 gene fragment in pSHAFT-GFP and its disruption with the Tp^R cassette derived from p34E-
437 TpTer, the resultant plasmid (pSHAFT-GFP-BCAL1709::TpTer) was introduced into AHA27
438 and candidate BCAL1709 mutants were selected by screening trimethoprim-resistant
439 exconjugants for the absence of GFP-mediated fluorescence. Among 50 ex-conjugants that
440 were screened in this way, 6 non-fluorescent recombinants were identified which were
441 subsequently verified as BCAL1709 mutants by PCR, as indicated by a ~900 bp increase in
442 the size of the DNA fragment amplified from the BCAL1709 locus due to the presence of the
443 Tp^R cassette (Figure 3C).

444 It should also be noted that use of the Tp^R cassette in allelic replacement
445 experiments is also applicable to *Burkholderia* strains that exhibit higher levels of intrinsic
446 resistance to trimethoprim, such as members of the *B. cenocepacia* ET-12 lineage, i.e.
447 strains J2315 and K56-2. Due to the highly active promoter located upstream of the *dfrB2*
448 ORF, expression of the gene is sufficiently strong to allow its selection in such strains by
449 increasing the concentration of trimethoprim in the medium, and we have used pSHAFT2 to
450 transfer Tp^R cassette-disrupted iron acquisition and type VI secretion system genes into
451 K56-2 (unpublished results).

452

453 4. Conclusion

454 To conclude, we have constructed a series of versatile suicide vectors and antibiotic
455 resistance cassettes that allow for the efficient and simple generation of marked mutants in
456 *Burkholderia* species. We have improved upon our original vector pSHAFT by reducing the
457 size of the Cm^R marker and increasing the availability of unique restriction site for cloning in
458 vectors pSHAFT2 and pSHAFT3. The versatility of this plasmid series has been further
459 enhanced by incorporating a fluorescent marker in pSHAFT-GFP to provide an alternative

460 means of distinguishing recombinants that have undergone allelic replacement events from
461 those in which the suicide vector remains integrated in the genome. All vectors described
462 here are a useful addition to the molecular toolkit required for the manipulation and
463 subsequent characterisation of important genes in not only *Burkholderia* species, but
464 potentially other Proteobacteria, such as enterobacteria and pseudomonads, where they
465 may be used in combination with the antibiotic-resistance cassettes described herein or
466 other available antibiotic-resistance markers.

467

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662 **Figure 1 Maps of novel antibiotic-cassette vectors.** The location of antibiotic resistance-
663 conferring genes, *bla* (ampicillin-resistance), *aphA2* (kanamycin-resistance), *catA2*
664 (chloramphenicol-resistance) and *dfrB2* (trimethoprim-resistance), and the plasmid ColE1
665 origin of replication (oriR) are indicated for plasmids p34E-Km (top), p34E-Cm2 (left) and
666 p34E-TpTer. Dual-cutting restriction sites that can be utilised to excise the antibiotic
667 resistance cassette are also shown, as are internal sites that can be used for determination
668 of the orientation of the cassette following insertion into a target gene. Restriction sites that
669 occur only once in each plasmid are shown in bold. Note that additional sites for *Ase*I, *Bsr*GI,
670 *Nhe*I and *Xmn*I occur in the backbone of all three vectors that for clarity are not shown.
671 Promoters for the cassette antibiotic-resistance genes are shown (P_{aph}, P_{cat}, P_{cS}). Maps
672 created with SnapGene® software (from GSL Biotech; available at snapgene.com).
673
674 **Figure 2 Maps of the gene replacement vectors of the pSHAFT-series utilised for**
675 **marked mutagenesis in *Burkholderia*.** The location of antibiotic-resistance conferring
676 genes, *bla* (ampicillin-resistance) and *catA2* (chloramphenicol-resistance), RP4 origin of
677 transfer (*oriT*), R6K origin of replication (*oriR6K*) and GFP-encoding gene (*gfp*) are indicated
678 for pSHAFT, (left), pSHAFT2 (top right), pSHAFT3 (centre right) and pSHAFT-GFP (bottom
679 right). The transcriptional orientation for each gene and restriction sites within the multiple
680 cloning site of each vector are shown. Additional restriction sites in pSHAFT flanking Ω-Cm
681 (dashed line) are also indicated. Restriction sites that occur only once in each plasmid are
682 shown in bold. Maps created with SnapGene® software (from GSL Biotech; available at
683 snapgene.com).
684
685 **Figure 3 Generation of marked mutants in *B. cenocepacia* using pSHAFT2 and**
686 **pSHAFT-GFP derivatives.** (A) $\geq$ 1.0 kb of DNA containing the target gene (or gene
687 fragment) is cloned into a pSHAFT vector and then disrupted by insertion of an antibiotic
688 resistance cassette, ensuring there is at least 0.5 kb of homology between the cloned DNA
689 target region and the chromosome on either side of the cassette. Following transfer of the
690 pSHAFT-derived construct into *Burkholderia*, double crossover recombinants are selected

691 for based on their resistance to the antibiotic specified by the antibiotic resistance cassette,
692 and either sensitivity to chloramphenicol (pSHAFT2 and pSHAFT3) or the absence of
693 fluorescence (pSHAFT-GFP). Candidate mutants are then verified by PCR using primers
694 that anneal to genomic sequences located either side of the region cloned into the allelic
695 replacement vector ('outside' primers), indicated as OPfor and OPrev. Drawn to scale. (B)
696 PCR screening of candidate H111-ΔBCAM0195::Tp mutants following allelic replacement
697 with pSHAFT2-ΔBCAM0195'::Tp. (C) PCR screening of candidate AHA27-BCAL1709::TpTer
698 mutants following allelic replacement with pSHAFT-GFP-BCAL1709::TpTer.

Figure 1.

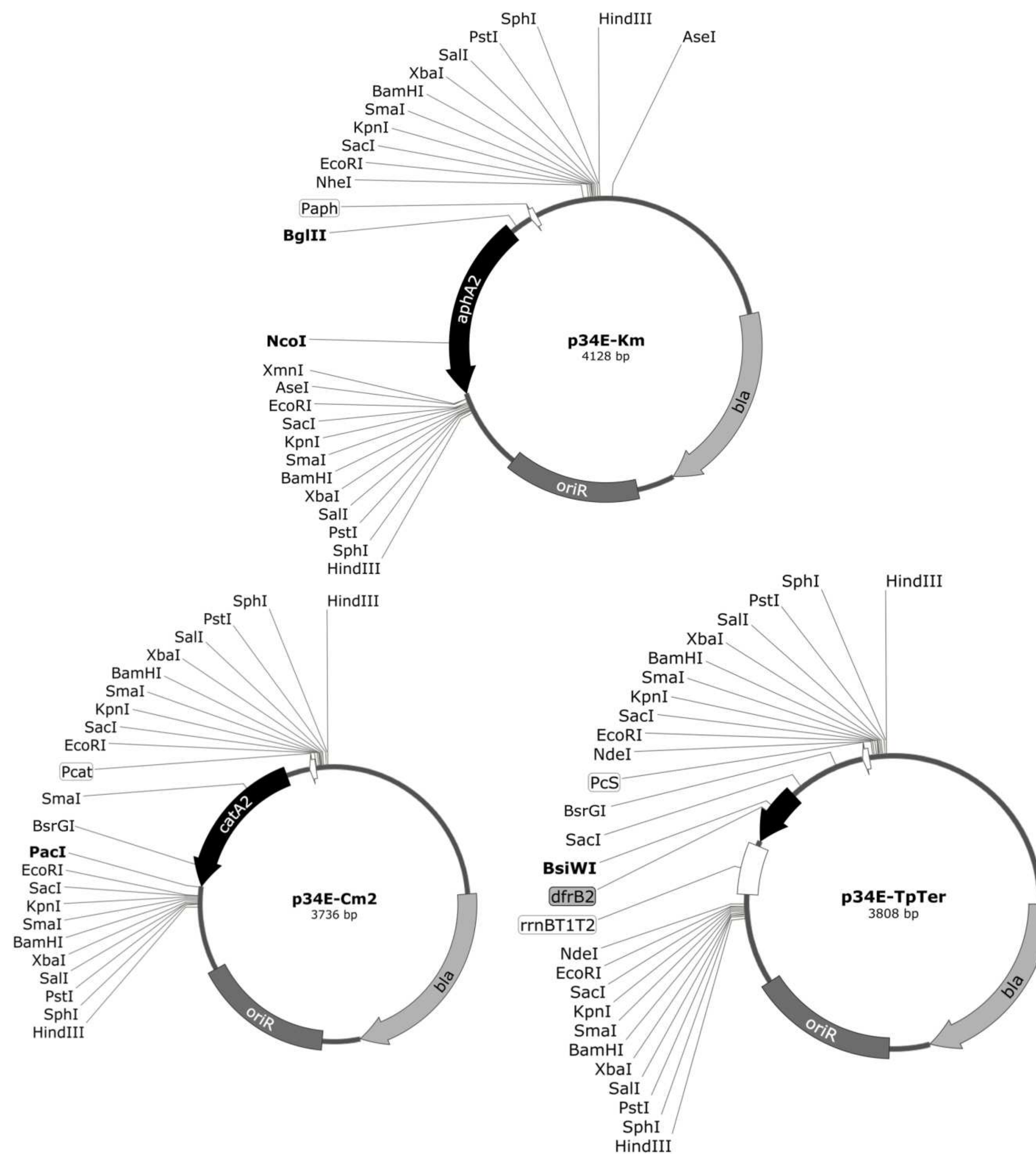


Figure 2.

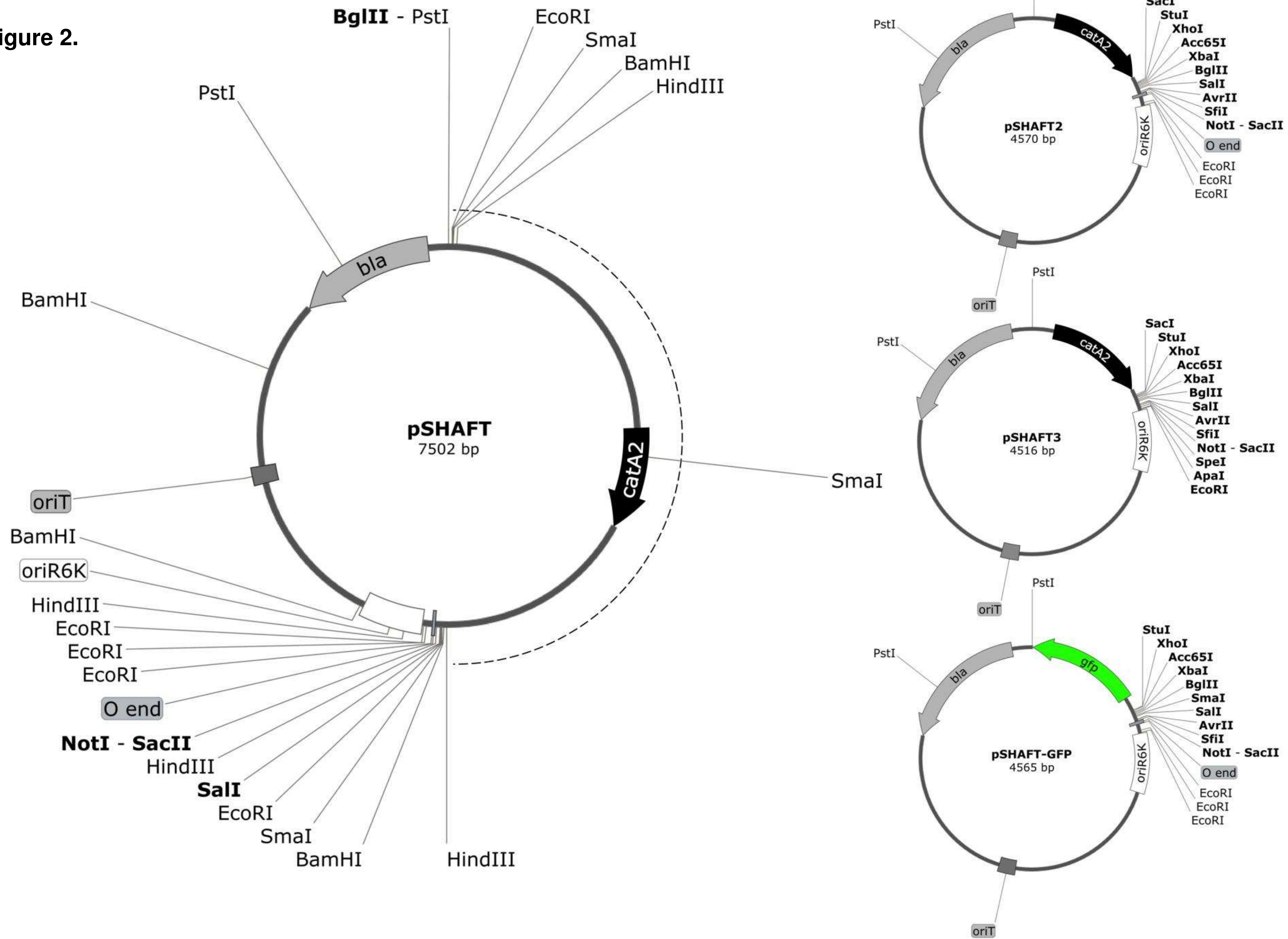
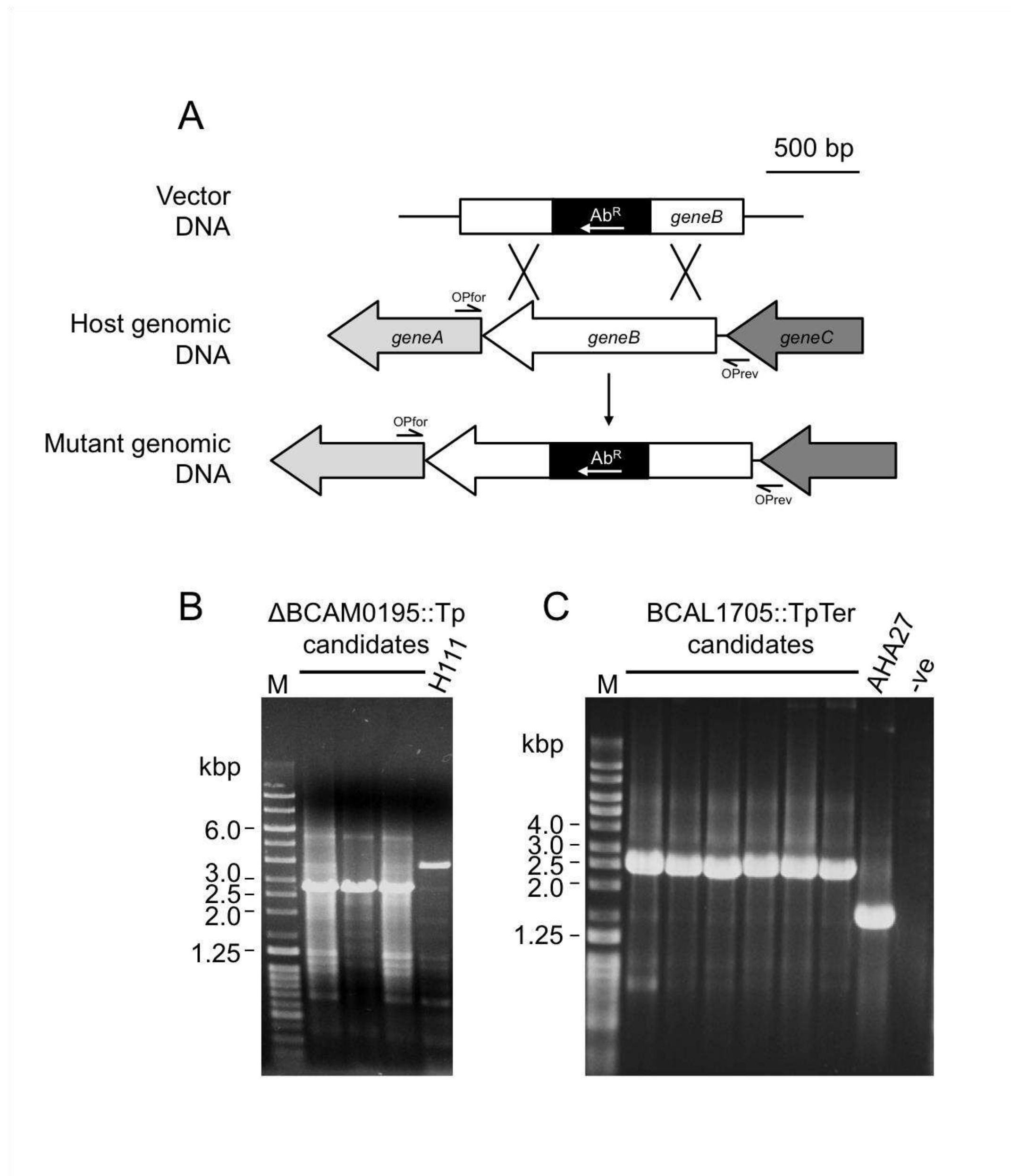


Figure 3.



Supplemental

Table S1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
***E. coli* strains**		
JM83	F^{-} *ara* Δ(l*ac-proAB*) *rpsL* *ϕ*80d*lacZ*ΔM15 (Sm^{R})	Yanisch-Perron et al., 1985
SM10(λpir)	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^{R})(λpir)	Simon et al., 1983
S17-1(λpir)	*thi proA hsdR recA* RP4-2-*tet*::Mu-1 *kan*::Tn7 integrant (Tp^{R}, Sm^{R}) (λpir)	Simon et al., 1983
***B. cenocepacia* strains**		
Pc715j	CF isolate, prototroph	McKevitt et al., 1989
H111	CF isolate, prototroph	Römling et al., 1994
J2315	CF isolate	Holden et al., 2009
K56-2	CF isolate, prototroph	Darling et al., 1998; Mahenthiralingam et al., 2000
AHA27	Pc715j-*pobA*::mini-Tn*5*CmlacZYA	Asghar et al., 2011
H111-BCAM0195::Tp	H111 with *dfrB2* cassette inserted in BCAM0195 (Tp^{R})	This study
AHA27-BCAL1709::TpTer	AHA27 with *dfrB2* cassette containing *rrnB* T1T2 terminators inserted in BCAL1709 (Tp^{R})	This study
***B. lata* strains**		
383	Soil isolate, prototroph (also known as (ATCC 17760, NCIB 9087, LMG 22485)	Stanier et al., 1966; Vanlaere et al., 2009

***B. thailandensis* strains**		
E264	Rice paddy isolate, prototroph	Brett et al.,1998
Plasmids		
pEA302T	*E. coli*-specific vector containing phage λ *c*I under P_{tac} with *rrnB* T1T2 terminators (Ap^R, Tc^R)	Amann et al., 1983
pBBR1MCS-1	Mobilizable BHR cloning vector, pBBR1-replicon (Cm^R)	Kovach et al., 1994
pUC18	*E. coli*-specific cloning vector (Ap^R)	Yanisch-Perron et al., 1985
pUC19	*E. coli*-specific cloning vector (Ap^R)	Yanisch-Perron et al., 1985
pUC18Ter	pUC18 containing *rrnB* T1T2 terminators (Ap^R)	This study
pUC19TpTer	pUC19 containing *dfrB2* gene fused to *rrnB* T1T2 terminators (Ap^R, Tp^R)	This study
pKNOCK-Km	Mobilizable suicide vector containing *aphA2* gene (Km^R)	Alexeyev, 1999
p34E-Tp	p34E containing *dfrB2* gene (Ap^R, Tp^R)	DeShazer and Woods, 1996
p34E-Km	p34E containing *aphA2* gene from pKNOCK-Km (Ap^R, Km^R)	This study
p34E-Cm2	p34E containing *catA2* gene from pSa with synthetic promoter (Ap^R, Cm^R)	This study
p34E-TpTer	p34E containing *dfrB2* gene fused to *rrnB* T1T2 terminators (Ap^R, Tp^R)	This study

pBHR1-GFP	pBHR1 containing *gfp* gene from pQBI-T7-GFP (Cm^R, Km^R)	(Stevens et al., 2005)
pSHAFT	Suicide vector derived from pUTmini-Tn*5*Cm containing deletion of *Bgl*II fragment harboring *tnp* gene and I end of mini-Tn*5*Cm, R6K-derived replicon, *ori*T$^+$(Ap^R, Cm^R)	(Shalom, 2002; Agnoli et al., 2006)
pSHAFT-GFP	pSHAFT with 3.7 kb Ω-Cm interposon replaced by *gfp* gene from pBHR1-GFP	This study
pSHAFT2	pSHAFT with 3.7 kb Ω-Cm region replaced by *catA2* gene driven by synthetic promoter and additional unique restriction sites at 3' end (Ap^R, Cm^R)	This study
pSHAFT3	pSHAFT2 derivative with deletion of two *Eco*RI sites, addition of *Apa*I and *Spe*I sites and removal of the mini-Tn*5* O end (Ap^R, Cm^R)	This study
pBBR1-BCAM0195'	pBBR1MCS-1 containing *B. cenocepacia* H111 BCAM0195 gene fragment, lacking 0.55 kbp at the 3' end (Cm^R)	This study
pBBR1-BCAM0195'::Tp	pBBR1-BCAM0195' with *dfrB2* gene replacing a 1.58 kb segment of BCAM0195' (Cm^R, Tp^R)	This study
pSHAFT2-BCAM0195'::Tp	pSHAFT2 containing the *Xho*I-*Xba*I BCAM0195'::Tp fragment from pBBR1-BCAM0195'::Tp (Ap^R, Cm^R, Tp^R)	This study
pSHAFT-GFP-BCAL1709	pSHAFT-GFP containing *B. cenocepacia* Pc715j BCAL1709 gene (Ap^R, Cm^R)	This study

pSHAFT-GFP-BCAL1709::TpTer	pSHAFT-GFP-BCAL1709 with *dfrB2-rrnB* T1T2 cassette inserted in BCAL1709 (Ap^R, Cm^R, Tp^R)	This study

Abbreviations: Ap^R, ampicillin-resistant; Cm^R, chloramphenicol-resistant; Km^R, kanamycin-resistant; Sm^R, streptomycin-resistant; Tc^R, tetracycline-resistant; Tp^R, trimethoprim-resistant; BHR, broad host range.

Table S2. Primers used in this study

Primer ID	Primer sequence[a,b]
p34E-Cmfor2	5'-GCGCGAATTCTTGACAATTAAGCCCGTATATGGTATTATTACTGAAT
p34E-Cmrev	5'-GCGCGAATTCCCGGATACGGTGGCTTAAAT
rrnBterfor	5'-CGCGGATCCAATTGAGAGTAGGGAACTGCCAGGCA
rrnBterrev	5'-GCGCAAGCTTCTCGAGGGTACCGAGCTCGAATTCTTGTAGATATGACGACAGGA
Tp(forward)	5'-GCGGAATTCAGGCCTCATATGCACGAACCCAGTTGACAT
Tp(reverse)	5'-CTGGCAGTTCCCTACTCTCTTAGGCCACACGTTCAAGTGC
rrn(R)	5'-GACAAGCTTAGGCCTCATATGGTAGATATGACGACAGGAAGAG
pSHOOTERfor2	5'-GCGCGGATCCTTGACAATTAAGCCCGTATATGGTATTATTACTGAAT
pSHOOTERrev	5'-GCGCGTCGACAAAGATCTAATCTAGAAAGGTACCAACTCGAGAAAGGCCTAAGAGCTCCCGGATACGGTGGCTTAAAT
pSHAFT3MCSfor	5'-GGCCGCAAACTAGTAAGGGCCCAAG
pSHAFT3MCSrev	5'-AATTCTTGGGCCCTTACTAGTTTGC
GFPfor	5'-GCGCGTCGACAACCCGGGAAAGATCTAATCTAGAAAGGTACCAACTCGAGAAAGGCCTTTGACATTTGCAGATTCGCCTTCTATAATAATTCGCCCTTCCCCTGTAGAAATAATTTTG
GFPrev	5'-CTTTGTTAGCAGCCGGATCC
BCAM0195for	5'-GCGCAAGCTTATGAGCGGCCTGCTCGATCA
BCAM0195rev	5'-GCGCGGATCCACGTCTTCACCACGCGGGTT
BCAL1709for	5'-GCGCTCTAGAGCTGCTGCAGTTCGAATACG
BCAL1709rev	5'-CGTCGCATTCGCGTAGTAGT
BCAL1709forOut	5'-CGGAAAACTTCGGACATGTG

BCAL1709revOut	5'-GCGAGGTCAGAACTTGTATG

[a]Sequences specifying restriction endonuclease cleavage sites are underlined.

[b]Sequences corresponding to the -35 and -10 elements of the artificial promoters introduced upstream of the *catA2* and *gfp* genes are enclosed in boxes. The former is an 'extended' -10 promoter (Bown et al., 1997).