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**A Reliable Method for the Selection and Confirmation of Transconjugants of Plant
Growth-Promoting Bacteria especially Plant-Associated *Burkholderia* spp.**

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28
29 **Abstract**

30 Selectable markers, e.g., antibiotic resistance, for conjugation experiments are not always
31 effective for slow-growing plant growth promoting bacteria such as *Burkholderia*. We
32 used PCAT medium containing Congo Red for selecting *Burkholderia* transconjugants.
33 This method allows for the reliable selection of transconjugants of these novel plant
34 growth-promoting bacteria.

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36 Keywords: Conjugation, Congo Red, PGPB, PCAT, Transconjugants

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38 The most frequently used method of introducing marker genes into the majority of Gram-
39 negative bacteria is via a triparental mating. However, selection and confirmation of
40 transconjugants is the most critical and sometimes most difficult part of conjugation
41 experiments. Use of minimal media aids the selection of the recipient cells
42 (transconjugants), but sometimes finding the proper medium is problematic. Similarly,
43 adding antibiotics to the selection medium helps in finding recipients, but *E. coli* cells
44 often pick up resistance, leading to false positives, especially when recipient cells grow
45 slowly. PCR, RAPD, RFLP, 16S rRNA gene sequencing, etc. are essential for
46 confirming the identity of the transconjugants (Golding et al., 2007; Phornphisutthimas et
47 al., 2007), but considerable time and money may be spent before learning that the
48 presumed transconjugants are *E. coli*. Here, we describe a reliable and cost-effective
49 method for the selection and confirmation of transconjugants of plant growth promoting
50 bacteria (PGPB), especially plant-associated *Burkholderia* spp.

52 The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* colonies
53 were grown routinely on LB (Luria-Bertani) medium at 37°C. The *Burkholderia* strains
54 were grown on LB minus NaCl and the remaining PGPB bacteria were grown on yeast
55 extract mannitol (YEM) agar at 28-30°C. The different media were supplemented with
56 tetracycline (10 µg/mL), rifampicin (20 µg/mL), or kanamycin (50 µg/mL) for growing
57 the various strains (Table 1). Congo Red (CR) was added to the media at 2.5 mg/L
58 (Kneen and Larue, 1983).

59 The PGPB recipients were transformed with the donor plasmids by a triparental mating
60 (Sambrook and Russell, 2001) whereas a modified version of the procedure (Glazebrook
61 and Walker, 1991) was used for the *Burkholderia* strains. LB minus NaCl plates were
62 employed for the mating step for the *Burkholderia* strains and LB for the others. The
63 mating plates were spread-plated with a 4:1 ratio of each *B. tuberum* strain to *E. coli*.
64 After one day, the mating plate was flooded with a sufficient quantity of 10 mM MgSO₄
65 to resuspend the cells. Dilutions were made of each suspension and a final one mL of the
66 cell suspension was spread-plated onto selection medium. The *E. coli* controls were also
67 streaked onto CR LB (Sambrook and Russell, 2001) agar plates.

68 Putative transconjugants of all the bacterial strains were initially selected on their
69 respective selection media. For example, the PGPB transconjugants were streaked onto
70 CR LB or YEM agar plates and the *Burkholderia* strains onto CR LB minus NaCl plates.
71 However, because the resulting *Burkholderia* “transconjugants” were red in color, we
72 switched to using CR PCAT (*Pseudomonas cepacia*, azelaic acid and tryptamine)
73 (Burbage and Sasser, 1982; Pallud et al., 2001) medium, with modifications, for the
74 selection plates. The tryptamine stock was diluted into DMSO instead of ethanol and the

1/24 aqueous suspension of chlorothalanil (Burbage and Sasser, 1982) was eliminated. The colors of the colonies growing on the CR-amended media were visually scored and photographed on either a black or white background. Confirmation of GFP fluorescence was performed by streaking transconjugants onto a glass slide and examining them on a Zeiss Axiophot microscope (BP 546, FT 580, LP 590).

For verification, genomic DNA was isolated from the putative transconjugants by a boiling lysis procedure (Sanders et al., 2010). The genomic DNA extractions were used as templates for amplification using the universal 16S primers (Sanders et al., 2010). The PCR products were run on agarose gels, excised, and prepared for sequencing by the UCLA GenoSeq Core. The sequences were processed through the NCBI BLASTN 16S ribosome RNA Database to assess identity.

Generation of transconjugants of *B. tuberum* is challenging in part because few tools have been developed for this microbe. Although reports describing GFP-labeled *B. tuberum* STM678 have been published (Chen et al., 2005; Elliott et al., 2007), the exact details of how the GFP was introduced in this species were presented only in Chen et al. (2003). In their paper, they employed a triparental mating strategy similar to ours, but with the final selection on YEM medium containing kanamycin and tetracycline after the mating had been performed on nitrocellulose. In our hands, we found that due to *B. tuberum*'s slower growth, it was difficult to select against *E. coli* on such a non-stringent medium. In our previous efforts where we used YEM medium and a rifampicin resistant *B. tuberum* strain and tried to generate transconjugants that carried a GFP-labeled promoter-probing transposon conferring kanamycin resistance, we ended up with *E. coli* colonies

that had acquired both rifampicin and kanamycin resistance. These colonies were verified as *E. coli* by PCR.

Before changing the protocol, we first tested the different bacterial strains on CR plates to see their phenotype. As reported by others (Berkhoff and Vinal, 1986; Styles and Flammer, 1991), we observed that *E. coli* colonies strongly absorbed CR (Fig. 1A-C). Wild-type and transconjugants of many PGPB recipient strains also turned various shades of red including *Agrobacterium tumefaciens* Ca18/GFP (Fig. 1D) and its parent strain (data not shown). Nakanishi et al. (1976) and Kneen and Larue (1983) reported that wild-type *Agrobacterium radiobacter* IFO13127 and *A. tumefaciens* A6, respectively, when grown on CR YEM agar plates, also turned bright red. On the other hand, *Azospirillum brasilense* UB1/GFP colonies were a lighter red color (data not shown) whereas *Pseudomonas putida* (Fig. 1E) and *P. fluorescens* (Fig. 1F) wild type and transconjugant strains carrying a mCherry plasmid had a pink or salmon color. Our results are consistent with the finding of Kneen and Larue (1983), who describe variable CR absorption by rhizobial and PGPB strains. In contrast, we observed that *B. tuberum* and *B. phymatum* colonies were white to light pink on plates containing CR (Fig. 2A, 2B). *B. tuberum pilA* and *exoY* mutants were also CR negative (data not shown).

Based on these observations, we used PCAT medium (Pallud et al., 2001), which has been utilized for *Burkholderia* species in soil, for selecting transconjugants. The mating mix was plated onto CR PCAT medium containing both antibiotics. Transconjugants took 4-5 days to appear and on this medium, they were small and CR negative (see insert in Fig. 2C). Colonies were further plate-purified on the same medium and subjected to additional testing including being examined for their CR phenotype on PCAT (Fig. 2C,

2D) and LB minus NaCl (Fig. 2E, 2F) containing rifampicin and kanamycin. The colonies were white to slightly pink, resistant to both antibiotics, and verified by PCR as either *B. phymatum* or *B. tuberum* strains (Table 2). They also grew on CR YEM minus CaCO₃, but longer incubation times resulted in the accumulation of a purple pigment in the medium (data not shown). Purpling occurs in response to acid-producing bacteria when CaCO₃ is omitted from the medium (Kneen and LaRue, 1983). As a further test, the *B. tuberum* mutants were inoculated onto bean (*Phaseolus vulgaris* cv. Negro Jamapa) plants and formed root nodules (data not shown). *B. phymatum* and *B. tuberum* mutant transconjugants also expressed GFP when viewed under epifluorescence (Fig. 3).

In summary, by using a combination of methods to confirm their identity, a highly selective medium, PCR, fluorescence, and nodulation, we found a reliable and cost-effective way to mobilize GFP with certainty into *Burkholderia* species. These strains will be useful for tracking the entry and infection of various *Burkholderia* strains and mutants as well as for biofilm experiments.

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Figure legends.

Figure 1. *E. coli* controls and PGPB grown on Congo Red plates. A. *E. coli* carrying pHC60 on LB. B. *E. coli* carrying pMP7604 on LB. C. *E. coli* carrying pUTgfp2x on LB. D. *Agrobacterium tumefaciens* Ca18 carrying pHC60 on LB. E. *Pseudomonas putida* PCL1445/mCherry (strain 1479) on LB. F. *P. fluorescens* WCS365/mCherry (strain 1700) on LB. Bar, 1 cm.

Figure 2. *Burkholderia* control and transconjugant strains grown on Congo Red-containing plates. A. *B. phymatum* STM815 on YEM. Bar, 1 cm. B. *B. tuberum* STM678 on PCAT. C. *B. tuberum* DUS833/GFP on PCAT. The insert shows the two original *B. tuberum* DUS833 colonies selected on a CR PCAT plate. D. *B. phymatum*/GFP on PCAT. E. *B. tuberum pilA*/GFP on LB - NaCl. Bar, 1 cm. F. *B. tuberum exoY*/GFP on LB - NaCl.

Figure 3. Transconjugants. Bars, 150 μ M. A. *B. tuberum exoY*/GFP. B. *B. tuberum pilA*/GFP. A thin film of agar covers the center of the cells in A and B. C. *B. phymatum* STM815/GFP. D. *B. tuberum* DUS833/GFP.

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1 Table 1. Characteristics of different bacterial strains and plasmids.

Bacterial strains and plasmids	Relevant characteristics	Reference or source
<i>Agrobacterium tumefaciens</i> Ca18	Wild type; plant growth promoting rhizobacteria	This work
<i>Pseudomonas fluorescens</i> WCS365	Wild type; excellent colonizer of tomato roots	Geels and Schippers, 1983
<i>Pseudomonas putida</i> PCL1445	Wild type; colonizer of grass roots. Produces lipopeptides putisolvin I and II	Kuiper et al., 2004
<i>Burkholderia phymatum</i> STM815 ^T	Wild type; nodulates Mimosoid legumes	Vandamme et al., 2002; Chen et al., 2005; Moulin et al., 2014
<i>Burkholderia tuberum</i> DUS833	Wild type; nodulates Papilionoid legumes.	Elliott et al., 2007
<i>Burkholderia tuberum</i> STM678 ^T	Wild type; nodulates Papilionoid legumes.	Moulin et al., 2001; Elliott et al., 2007; Vandamme et al., 2002
<i>Burkholderia tuberum pilA</i>	<i>pilA</i> - mutant, Rif ^r	M.R. Lum
<i>Burkholderia tuberum exoY</i>	<i>exoY</i> - mutant, Rif ^r	M.R. Lum
Plasmids		
pRK2013	Helper plasmid for triparental mating, Kan ^r	Figurski and Helinski, 1979
pRK600	Helper plasmid for <i>Burkholderia</i> mating Cam ^r	Christensen et al., 1998
pHC60	Broad host-range cloning vector for Gram-negative bacteria harboring green fluorescent protein (GFP), Tet ^r	Cheng and Walker, 1998
pMP7604	Broad host-range cloning vector for Gram-negative bacteria harboring mCherry gene under the control of the <i>tac</i> promoter, Tet ^r	Lagendijk et al., 2010
pUTGFP2x	GFP donor plasmid, Amp ^r , Kan ^r	Unge et al., 1997, 1998

Table 2. Best matches for transconjugants following PCR with 16S Primers

Putative Transconjugant	Identity	Value	Accession
Trial 1 <i>B. phymatum</i> /GFP	<i>B. phymatum</i> STM815	97%	NR 027555.1
Trial 2 <i>B. phymatum</i> /GFP	<i>B. phymatum</i> STM815	99%	NR 027555.1
<i>B. tuberum</i> <i>exoY</i> /GFP	<i>B. tuberum</i> STM678	99%	NR 027554.1
<i>B. tuberum</i> <i>pilA</i> /GFP	<i>B. tuberum</i> STM678	100%	NR 027554.1
<i>B. tuberum</i> DUS833	<i>B. tuberum</i> STM678	95%	NR 027554.1

Figure 1

Figure 2

Figure 3

Graphical Abstract