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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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ABSTRACT

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

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

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

The PGPB recipients were transformed with the donor plasmids by a triparental mating (Sambrook and Russell, 2001) whereas a modified version of the procedure (Glazebrook and Walker, 1991) was used for the *Burkholderia* strains. LB minus NaCl plates were employed for the mating step for the *Burkholderia* strains and LB for the others. The mating plates were spread-plated with a 4:1 ratio of each *Burkholderia* strain to *E. coli*. After one day, the mating plate was flooded with a sufficient quantity of 10 mM $MgSO_4$ to resuspend the cells. Dilutions were made of each suspension and a final 1 mL of the cell suspension was spread-plated onto selection medium. The *E. coli* controls were also streaked onto CR LB (Sambrook and Russell, 2001) agar plates.

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Table 1
Characteristics of different bacterial strains and plasmids.

	Relevant characteristics	Reference or source
Bacterial strains and plasmids		
Agrobacterium tumefaciens Ca18	Wild type; plant growth promoting rhizobacteria	This work
Pseudomonas fluorescens WCS365	Wild type; excellent colonizer of tomato roots	Geels and Schippers (1983)
Pseudomonas putida PCL1445	Wild type; colonizer of grass roots. Produces lipopeptides putisolvin I and II	Kuiper et al. (2004)
Burkholderia phymatum STM815^{T}	Wild type; nodulates Mimosoid legumes	Elliott et al. (2007a); Moulin et al. (2014)
Burkholderia tuberum DUS833	Wild type; nodulates Papilionoid legumes.	Elliot et al. (2007b)
Burkholderia tuberum STM678 ^{T}	Wild type; nodulates Papilionoid legumes.	Moulin et al. (2001); Chen et al. (2005); Elliot et al. (2007b)
Burkholderia tuberum pilA	*pilA*-mutant, Rif^{r}	M.R. Lum
Burkholderia tuberum exoY	*exoY*-mutant, Rif ^{r}	M.R. Lum
Plasmids		
pRK2013	Helper plasmid for triparental mating, Kan^{r}	Figurski and Helinski (1979)
pRK600	Helper plasmid for *Burkholderia* 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 *tac* promoter, Tet^{r}	Lagendijk et al. (2010)
pUTGFP2x	GFP donor plasmid, Amp^{r}, Kan^{r}	Unge et al. (1997, 1998)

Putative transconjugants of all the bacterial strains were initially selected on their respective selection media. For example, the PGPB transconjugants were streaked onto CRLB or YEM agar plates and the *Burkholderia* strains onto CR LB minus NaCl plates. However, because the resulting *Burkholderia* "transconjugants" were red in color, we switched to using CR PCAT (*Pseudomonas cepacia*, azelaic acid and tryptamine) (Burbage and Sasser, 1982; Pallud et al., 2001) medium, with modifications, for the 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.

Burkholderia tuberum STM678 and *B. phymatum* STM815 were originally described by Moulin et al. (2001) and Vandamme et al. (2002).

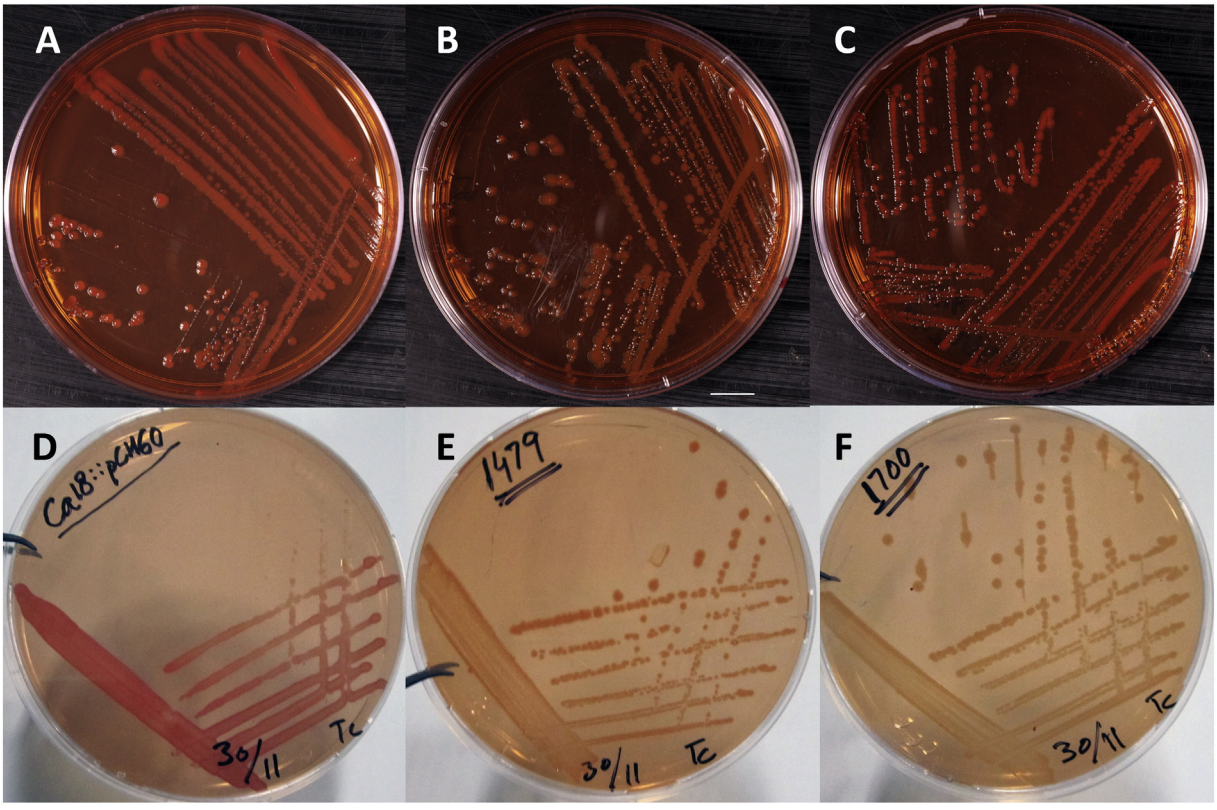

Fig. 1. *E. coli* controls and PGPB grown on Congo Red plates. A. *E. coli* carrying pHC60 on LB. B. *E. coli* carrying pMP7604 on LB. C. *E. coli* carrying pUTgfp2x on LB. D. *Agrobacterium tumefaciens* Ca18 carrying pHC60 on LB. E. *Pseudomonas putida* PCL1445/mCherry (strain 1479) on LB. F. *P. fluorescens*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

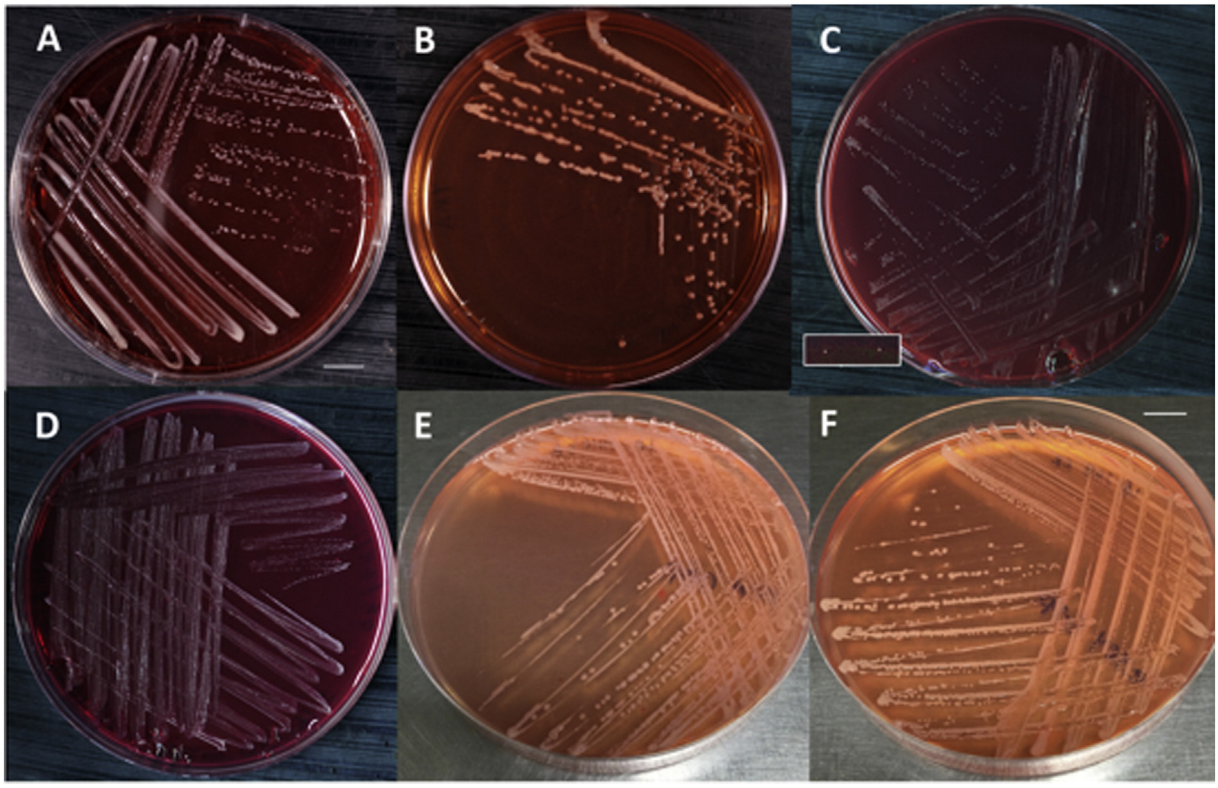

Fig. 2. *Burkholderia* control and transconjugant strains grown on Congo Red-containing plates. A. *B. phymatum* STM815 on YEM. Bar, 1 cm. *B. tuberum* STM678 on PCAT. C. *B. tuberum* DUS833/GFP on PCAT. The inset shows the two original *B. tuberum* DUS833 colonies selected on a CR PCAT plate. D. *B.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

Generation of transconjugants of *B. tuberum* in particular is challenging in part because few tools have been developed for this microbe. Although reports describing GFP-labeled *B. tuberum* STM678 and *B. phymatum* STM815 have been published (Chen et al., 2005; Elliot et al., 2007a,b), the exact details of how the GFP was introduced in these 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 phenotypes. 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 *Pseudomonas 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 *Burkholderia phymatum* colonies were white to light pink on plates containing CR (Fig. 2A, B). *B. tuberumpilA* and its *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 inset 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, D) and LB minus NaCl (Fig. 2E, F) 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 $CaCO_3$, 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 $CaCO_3$ 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).

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

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

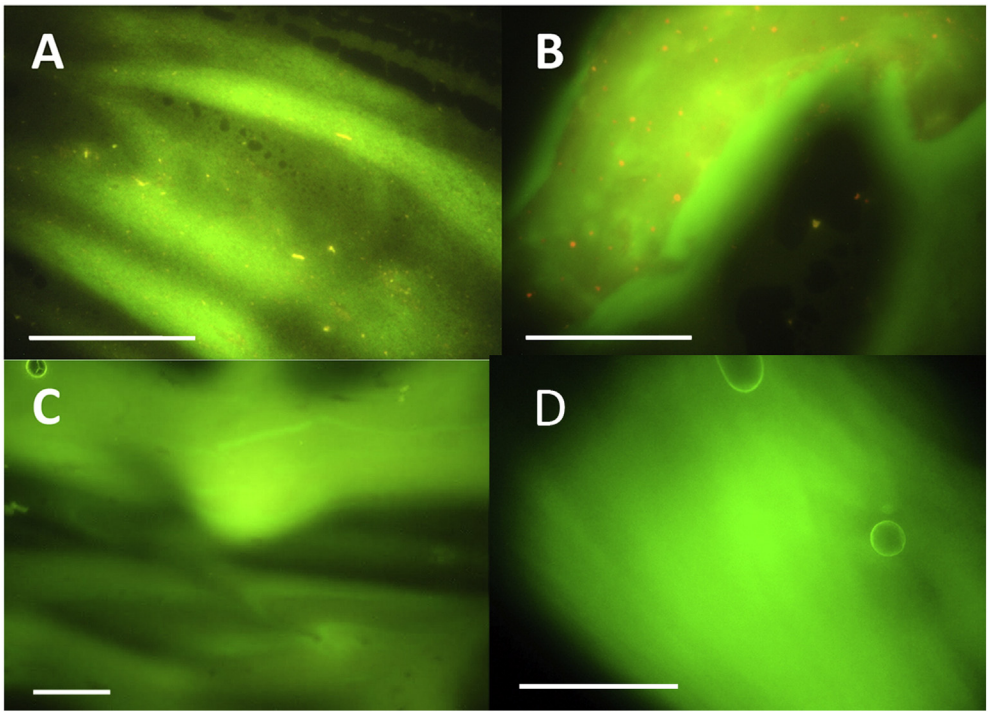

Fig. 3. Transconjugants. Bars, 150 μM. A. *B. tuberum exoY*/GFP. B. *B. tuberum pilA*/GFP. A thin film of agar covers the center of the cells in A and B. C. *B. phymatum* STM815/GFP. D. *B. tuberum* DUS833/GFP.

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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References

Berkhoff, H.A., Vinal, A.C., 1986. Congo Red medium to distinguish between invasive and non-invasive *Escherichia coli* pathogenic for poultry. Avian Dis. 30, 117–121.

Burbage, D.A., Sasser, M., 1982. A medium selective for *Pseudomonas cepacia*. Phytopathol. Abstr. 72, 706.

Chen, W.-M., James, E.K., Prescott, A.R., Kierans, M., Sprent, J.I., 2003. Nodulation of *Mimosa* spp. by the β-proteobacterium *Ralstonia taiwanensis*. Mol. Plant Microbe Interact. 12, 1051–1061.

Chen, W.-M., de Faria, S.M., Straliotto, R., Pitard, R.M., Simões-Araùjo, J.L., Chou, J.-H., Chou, Y.-J., Barrios, E., Prescott, A.R., Elliott, G.N., Sprent, J.I., Young, J.P.W., James, E.K., 2005. Proof that *Burkholderia* strains form effective symbioses with legumes: a study of novel *Mimosa*-nodulating strains from South America. Appl. Environ. Microbiol. 71, 7461–7471.

Cheng, H.P., Walker, G.C., 1998. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. 180, 5183–5191.

Christensen, B.B., Sternberg, C., Andersen, J.B., Eberl, L., Møller, S., Givskov, M., Molin, S., 1998. Establishment of new genetic traits in a microbial biofilm community. Appl. Environ. Microbiol. 64, 2247–2255.

Elliott, G.N., Chen, W.M., Chou, J.H., Wang, H.C., Sheu, S.Y., Perin, L., Reis, V.M., Moulin, L., Simon, M.F., Bontemps, C., Sutherland, J.M., Bessi, R., de Faria, S.M., Trinick, M.J., Prescott, A.R., Sprent, J.I., James, E.K., 2007a. Burkholderia phymatum is a highly effective nitrogen-fixing symbiont of Mimosa spp. and fixes nitrogen ex planta. New Phytol 173, 168–180.

Elliot, G.N., Chen, W.M., Bontemps, C., Chou, J.-H., Young, J.P.W., Sprent, J.I., James, E.K., 2007b. Nodulation of *Cyclopia* spp. (Leguminosae, Papilionoideae) by *Burkholderia tuberum*. Ann. Bot. 100, 1403–1411.

Figurski, D.H., Helinski, D.R., 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. U. S. A. 76, 1648–1652.

Geels, F.P., Schippers, B., 1983. Reduction of yield depressions in high frequency potato cropping soil after seed tuber treatments with antagonistic fluorescent *Pseudomonas* spp. Phytopathologica 108, 207–214.

Glazebrook, J., Walker, G.C., 1991. Genetic techniques in *Rhizobium meliloti*. Methods Enzymol. 204, 398–418.

Golding, G.R., Olson, A.B., Doublet, B., Cloeckaert, A., Christianson, S., Graham, M.R., Mulvey, M.R., 2007. The effect of the *Salmonella* genomic island 1 on *in vitro* global gene expression in *Salmonella enterica* serovar *Typhimurium* LT2. Microbes Infect. 9, 21–27.

Kneen, B.E., Larue, T.A., 1983. Congo Red absorption by *Rhizobium leguminosarum*. Appl. Environ. Microbiol. 45, 340–342.

Kuiper, I., Lagendijk, E.L., Pickford, R., Derrick, J.P., Lamers, G.E.M., Thomas-Oates, J.E., Lugtenberg, B.J.J., Bloemberg, G.V., 2004. Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. Mol. Microbiol. 51, 97–113.

Lagendijk, E.L., Validov, S., Lamers, G.E.M., de Weert, S., Bloemberg, G.V., 2010. Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. FEMS Microbiol. Lett. 305, 81–90.

Moulin, L., Munive, A., Dreyfus, B., Boivin-Masson, C., 2001. Nodulation of legumes by members of the beta-subclass of Proteobacteria. Nature 411, 948–950.

Moulin, L., Klonowska, A., Bournaud, C., Booth, K., Vriezen, J., Melkonian, R., James, E.K., Young, J.P., Bena, G., Hauser, L., Land, M., Kyrpides, N., Bruce, D., Chain, P., Copeland, A., Pitluck, S., Woyke, T., Lizotte-Waniewski, M., Bristow, J., Riley, M., 2014. Complete genome sequence of *Burkholderia phymatum* STM815T, a broad host range and efficient nitrogen-fixing symbiont of *Mimosa* species. Stand. Genomic Sci. 9, 763–777.

Nakanishi, I., Kimura, K., Suzuki, T., Ishikawa, M., Banno, I., Sakane, T., Harada, T., 1976. Demonstration of curdlan-type polysaccharide and some other β-1,3-glucan in microorganisms with aniline blue. J. Gen. Appl. Microbiol. 22, 1–11.

Pallud, C., Viallard, V., Balandreau, J., Normand, P., Grundmann, G., 2001. Combined use of a specific probe and PCAT medium to study *Burkholderia* in soil. J. Microbiol. Methods 47, 25–34.

Phornphisutthimas, S., Thamchaipenet, A., Panijpan, B., 2007. Conjugation in *Escherichia coli*—a laboratory exercise. Biochem. Mol. Biol. Educ. 35, 440–445.

Sambrook, J., Russell, D., 2001. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanders, E.R., Miller, J.H., Herbold, C., 2010. I, Microbiologist: A Discovery-based Course in Microbial Ecology and Molecular Evolution. ASM Press, Washington, D.C.
Styles, D.K., Flammer, K., 1991. Congo Red binding of *Escherichia coli* isolated from the cloacae of psittacine birds. Avian Dis. 35, 46–48.
Unge, A., Tombolini, R., Möller, A., Jansson, J.K., 1997. Optimization of GFP as a marker for detection of bacteria in environmental samples. In: Hastings, J.W., Kricka, L.J., Stanley, P.E. (Eds.), Bioluminescence and Chemiluminescence: Molecular Reporting with Photons. John Wiley & Sons, USA, New York, pp. 391–394.
Unge, A., Tombolini, R., Davey, M.E., de Bruijn, F.J., Jansson, J.K., 1998. GFP as a marker gene. In: de Bruijn, F.J., van Elsas, J.D., Akkermans, A.D.L. (Eds.), Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 1–16.
Vandamme, P., Goris, J., Chen, W.-M., de Vos, P., Willems, A., 2002. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. Syst. Appl. Microbiol. 25, 507–512.