**An improved conjugation method for *Pseudomonas syringae***

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**Abstract**

In order to achieve saturating transposon mutagenesis of the genome of plant pathogenic strains of *Pseudomonas syringae* we needed to improve plasmid conjugation frequency. Manipulation of the growth stage of donor and recipient cells allowed the required increase in frequency and facilitated conjugation of otherwise recalcitrant strains.

**Key words** *Pseudomonas*; conjugation; TnSeq

*Strains of Pseudomonas syringae* (*P.s.*) cause a wide range of economically important plant diseases worldwide. A number of *P. s. pathovars* from distinct phylogroups cause bacterial canker of cherry (*Prunus avium*) including *P. syringae* pv. *morsprunorum* (*Psm*) races 1 and 2 and *P. syringae* pv. *syringae* (*Pss*) (Hulin et al., 2020). Bioinformatic analyses have identified shared sets of genes putatively involved in the pathogenicity of *Psm* and *Pss* on cherry (Hulin et al., 2018). As a functional screen for genes in *Psm* and *Pss* regulating colonisation of, and persistence in, woody tissue we initiated an unbiased approach using saturating transposon mutagenesis. We proposed to use the method of TnSeq (transposon mutagenesis with next-generation sequencing) (Wetmore et al., 2015), which requires a high density of transposon insertion into the genome.

To achieve saturating mutagenesis a high conjugation frequency needs to be achieved for the transfer of the plasmid containing the transposon into the recipient *Pseudomonas* strain. Using routine protocols for the cherry pathogens we grew overnight cultures of *Pss* 9644, *Psm* R2 MH001 (formally R2 leaf) and R1 5244 (Hulin et al., 2018) and *E. coli* APA752 containing the *mariner* plasmid pKMW3 (Wetmore et al., 2015), cultured in LB broth for 18 h at 25°C or 37°C for *E. coli* respectively. One ml of each overnight culture was diluted 1 in 10 and grown for 6 h to reach log phase (0.8 OD600) (Sup Fig. 1). Equal cell numbers (500 µl each of 0.8 OD600) of *Pseudomonas* strains and *E. coli* were combined and allowed to conjugate on 0.45µm nitrocellulose filters (Millipore) placed on LB agar plates supplemented with 0.0625 mg/mL diaminopimelate (in sterile distilled water) at 30°C. After 6 h cells were scraped off the filters, serially diluted and plated onto KB agar with 25µg/mL kanamycin (Kan) and either 100 µg/ml nitrofurantoin (Nf, in DMSO) for *Pss* 9644 and *Psm* R2 MH001 or 100 µg/ml rifampicin (Rif, in methanol) for *Psm* R1 5244. Using this standard protocol we achieved a maximum of 2.6x102 transconjugants (CFU) per ml of conjugation mixture (Fig.1)which was insufficient to allow adequate saturation of the genome required for TnSeq screens (Wetmore *et al*. 2015). We therefore repeated the procedure but increased incubation time on the conjugation plates to 24h which increased the number of transconjugants 100-fold to a maximum of 1.5x104 CFU/ml for *Psm* R2 MH001. However, the conjugation frequency was much lower in *Psm* R1 5244 and *Pss* 9644 rendering it difficult to proceed with mapping the transposon mutant library and *in planta* experiments.

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**Figure 1. Effect of varying the time for conjugation on the frequency of transfer of the *mariner* plasmid pKMW3 into *Pseudomonas* strains using the Wetmore et al. (2015) method.** Extension of the routine 6h incubation on conjugation plates to 24h led to a statistically significant increase in conjugation frequency in all three strains, indicated by \* above the bars, as determined by a Student’s t-test (p<0.05). Data show means of three replicates ± standard error of mean (SEM) and are displayed as log10 cfu per ml of conjugation mixture, using strains of *Pseudomonas syringae* pv. *morsprunorum* R2 (*Psm*); *P. syringae* pv. *syringae* (*Pss*).

We next examined the effect of changing the growth stage of the donor and recipient. We used a combination of donor and recipient in stationary phase with an OD600 1.5 (18 h) and log phase with an OD600 0.8 (6 h) (Fig. 2), with a 24h conjugation incubation time. The conjugation frequency of pKMW3 into *Pseudomonas* strains using the donor cells in log phase and the recipient cells in stationary phase resulted in a ~1000 fold increase in conjugation frequency to a maximum of 2.7x107 CFU/ml (Fig.2). This new conjugation frequency was considered adequate to make the TnSeq libraries. To confirm that plasmid pKMW3 had transferred to the transconjugant cells, we amplified a section of the transposon using a standard PCR protocol with primers pKMW3F-5’GATGTCCACGAGGTCTCT3’, pKMW3R-5’GTCGACCTGCAGCGTAC3’ (Wetmore et al., 2015). A region of 100 bp was obtained in ten randomly selected transconjugants (data not shown).

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**Figure 2. Effect of varying the growth stage of donor (D) and recipient (R) cells on the frequency of conjugation of the *mariner* plasmid pKMW3 into *Pseudomonas* strains.** Incubation for log phase and stationary phase multiplication was for 6h (OD600 0.8) and 18h (OD600 1.5) respectively using strains of *Pseudomonas syringae* pv. *morsprunorum* R2 (Psm) and *P. syringae* pv. *syringae* (Pss). Using a combination of log phase (LP) donor cells and stationary phase (SP) recipient cells resulted in a statistically significant increase in conjugation frequency for all three strains as indicated by the \*, determined by a within-strain comparison of means by a Student’s t-test (p<0.05). Data show means of three replicates ± standard error of mean (SEM) and are displayed as log10 CFU/ml of conjugation mixture.

To expand this study, we tested our revised protocol on other *P. syringae* strains that have previously exhibited low conjugation rates. We used *P. syringae* pv. *phaseolicola* (*Pph*) 1448A (Joardar et al*.,* 2006), a good conjugator as a control and *Pph* 1302A (Taylor et al*.,* 1996), which has been very recalcitrant to plasmid conjugation in the past. We also tested additional recalcitrant strains - *P. syringae* RMA1, a pathogen of *Aquilegia vulgaris* (Hulin et al*.,* 2018) and the cherry pathogen *Pss* 9097 (Hulin et al*.,* 2018) (Fig. 3). The new method allowed transconjugants to be obtained at a reasonable frequency (1302A 4.7x103 CFU/ml; RMA1 2.1x103 CFU/ml; 9097 1.7x102 CFU/ml) with strains that had very low or no transconjugants using the original Wetmore et al. (2015) method.

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**Fig. 3. Effects of varying incubation period on conjugation plates and the growth phase of donor and recipient cells on the frequency of conjugation of the *mariner* plasmid pKMW3 into diverse *Pseudomonas* strains.** The combination of log phase donor cells and stationary phase recipient cells resulted in a statistically significant increase in conjugation frequency for all four *Ps* strains; isolates of*P. syringae* pv. *phaseolicola* (*Pph*); *P. syringae* (*Ps*) and *Pseudomonas syringae* pv. *syringae* (*Pss*). *Pph* 1302A, *Pss* 9097 produced no transconjugants at 6h and *Ps* RMA1 none at 6 or 24h. Statistical significance as indicated by the \* was determined by a within-strain comparison of means using a Student’s t-test (p<0.05), means are given of three replicates ±SEM and are displayed as log10 CFU/ml of conjugation mixture.

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Data underlying this article can be accessed at http://researchdata.uwe.ac.uk/583/

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