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DOI link to the version of record on the publisher's website

Neale, H.C., Hulin, M.T., Harrison, R.J., Jackson, R.W., Mansfield, J.W. and Arnold, D.L. 2020. An improved conjugation method for Pseudomonas syringae. *Journal of Microbiological Methods*, 106025.

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 OD_{600}) (Sup Fig. 1). Equal cell numbers (500 µl each of 0.8 OD_{600}) 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 2.6×10^2 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 1.5×10^4 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.

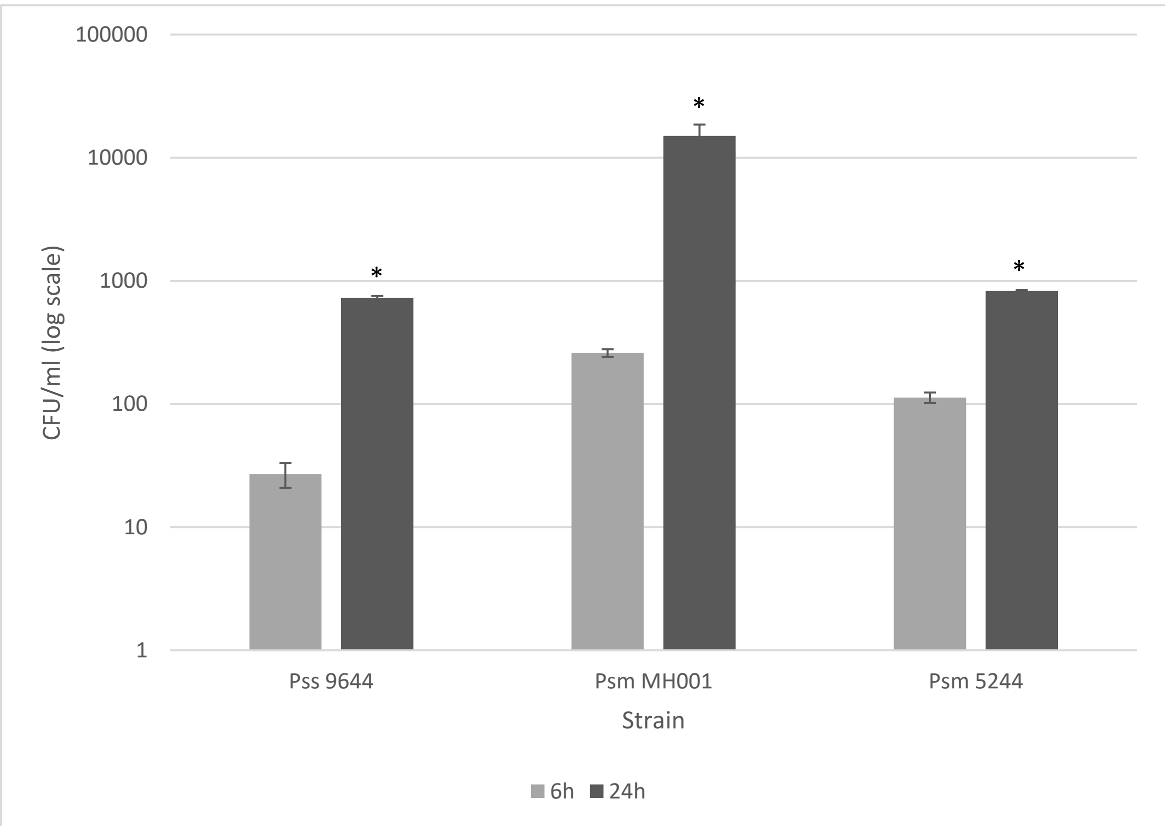

Figure 1. Effect of varying the time for conjugation on the frequency of transfer of the *mariner* plasmid pKMW3 into *Pseudomonas* strains using the Wetmore

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 $\log_{10}$ 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 OD_{600} 1.5 (18 h) and log phase with an OD_{600} 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 2.7×10^{7} 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).

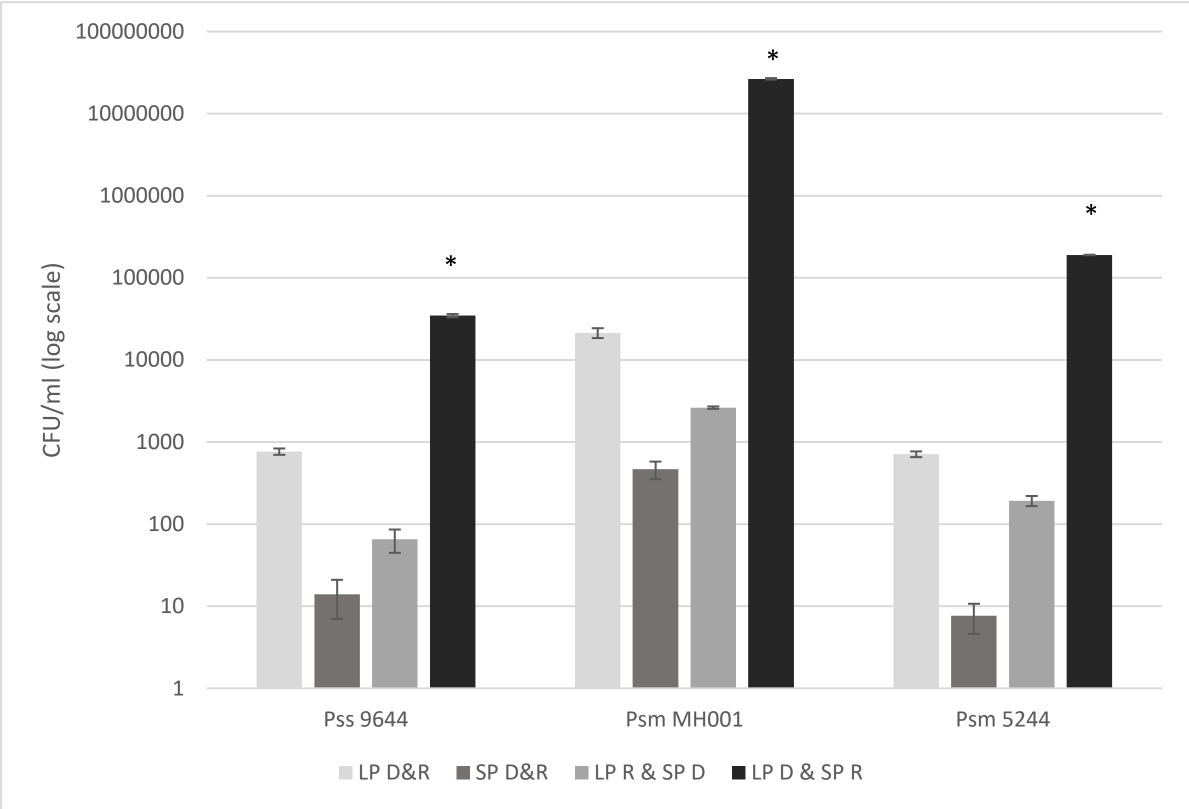

Figure 2. Effect of varying the growth stage of donor (D) and recipient (R) cells on the frequency of conjugation of the *mariner* plasmid pKMW3 into *Pseudomonas* strains. Incubation for log phase and stationary phase multiplication was for 6h (OD_{600} 0.8) and 18h (OD_{600} 1.5) respectively using 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 $\log_{10}$ 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 4.7x10^3 CFU/ml; RMA1 2.1x10^3 CFU/ml; 9097 1.7x10^2 CFU/ml) with strains that had very low or no transconjugants using the original Wetmore et al. (2015) method.

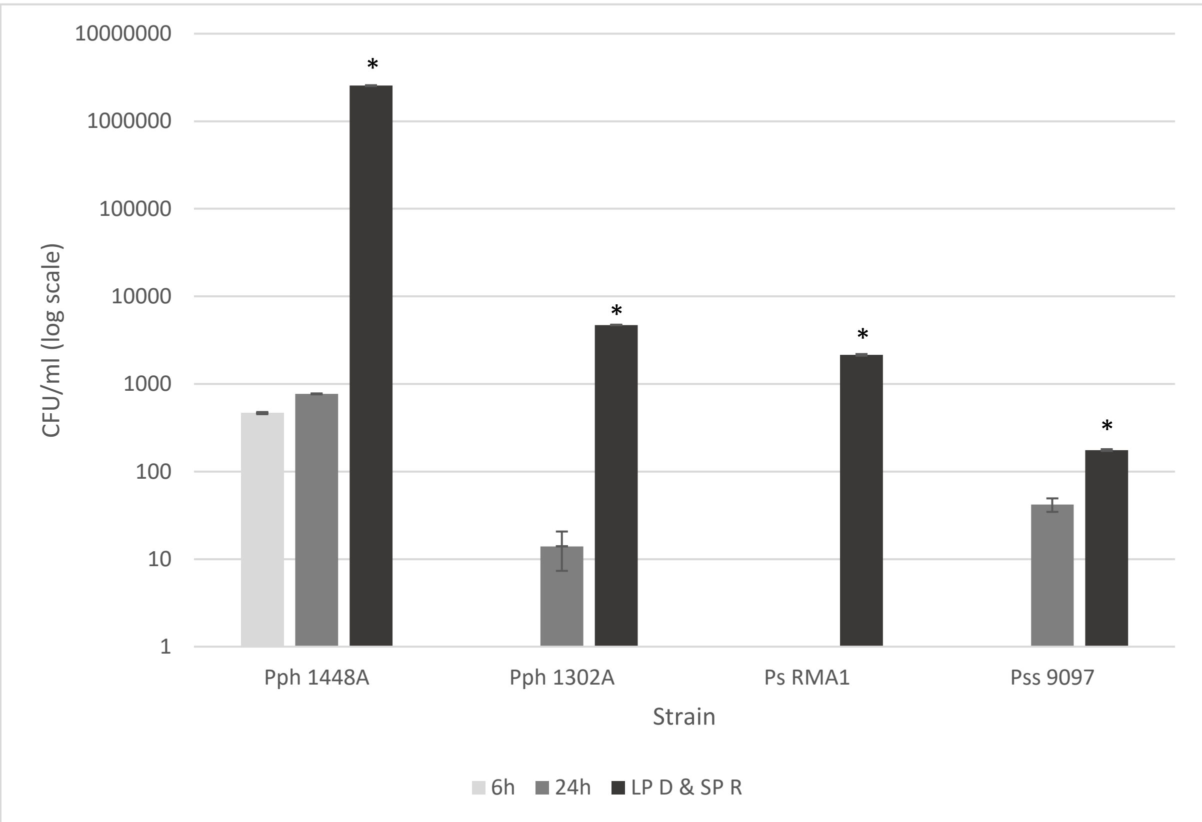

Fig. 3. Effects of varying incubation period on conjugation plates and the growth phase of donor and recipient cells on the frequency of conjugation of the *mariner* plasmid pKMW3 into diverse *Pseudomonas* strains. The combination of log phase donor cells and stationary phase recipient cells resulted 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 $\log_{10}$ CFU/ml of conjugation mixture.

Acknowledgments

We thank Adam Deutschbauer (Lawrence Berkeley National Laboratory, Berkeley, California, USA) for providing us with *mariner* vector pKMW3. This work was carried out under the Department for Environment, Food and Rural Affairs' Plant Health and Seeds Inspectorate license number 51049/202078/1. This research was funded by grants from the Biotechnology and Biological Sciences Research Council, United Kingdom (BB/P005705/1 and EMR grant number BB/ P006272/1).

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

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