

Note

## Construction and validation of a neutrally-marked strain of *Pseudomonas fluorescens* SBW25

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### Abstract

Neutrally marked bacterial strains are useful in many experimental evolution and molecular ecology studies to assess the relative fitness of a given strain. Here we describe the construction and validation of a neutral marker for the model organism *Pseudomonas fluorescens* SBW25. The marked strain, called SBW25-*lacZ*, was created by integrating a promoterless '*lacZ*' into the defective prophage locus of the SBW25 chromosome. Fitness assays conducted in various laboratory media and *in planta* revealed that the fitness levels of SBW25-*lacZ* were comparable with the wild-type ancestor.

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Fitness describes the overall ability of an organism to survive and reproduce in a given environment. It can be measured in bacteria by comparing the growth of two bacterial strains competing for resources in the same environment. Bacterial fitness is expressed as relative fitness, which is calculated on the basis of the ratios between the initial and final numbers of the two competitor strains (Lenski, 1991). Neutrally marked bacterial strains are important in bacterial genetics and experimental evolution because they allow simple examination of the fitness of genetically modified or evolved mutant strains relative to wild type. To minimize the systematic experimental errors and to easily distinguish one competitor strain from the other, a visual marker (e.g. a gene encoding a product that can be assayed via a colorimetric method) is often used. To be useful for experimental evolution studies, the marker should be stably maintained in the bacterial strain and its presence should not interfere with the growth rate of the strain (i.e., the marker should be neutral with respect to effects on fitness).

*Pseudomonas fluorescens* SBW25 is a Gram-negative bacterium originally isolated from the phytosphere of field-grown sugar beet (Bailey et al., 1995). As one of the model organisms

of plant-growth promoting rhizobacteria (PGPR), the genome of *P. fluorescens* SBW25 has been sequenced and gene functions are being extensively investigated. SBW25 is also an important model organism for experimental evolution and molecular ecology (Rainey and Travisano, 1998; Buckling et al., 2003; MacLean et al., 2005; Fukami et al., 2007). In a previous study, SBW25 was labelled (singly or in combination) by integration of three marker genes (*lacZY*, *aph-1*, and *xylE*) into different *loci* of the SBW25 genome (Bailey et al., 1995). The marked strains were successfully used to monitor plasmid gene transfers in the fields. However, these marked strains displayed decreased environmental fitness compared with the unmodified wild-type strain in maturing plants (De Leij et al., 1998). One strain (SBW25EeZY-6KX) was subjected to the same relative fitness assay described below and it showed a severe fitness decrease in sugar beet seedlings (see below). A pantothenate auxotrophic mutant (SBW25 $\Delta$ *panB*; Rainey, 1999) has been successfully used to determine bacterial fitness in microcosm experiments (Rainey and Travisano, 1998). This mutant produces noticeably smaller colonies on indicator plates with a low concentration of pantothenate and competes equally well with the wild-type ancestor when excess pantothenate is provided in the medium. However, a decrease in fitness was observed with SBW25 $\Delta$ *panB* during extended periods of growth (>7 days) (MacLean et al., 2005). In addition, SBW25 $\Delta$ *panB* is

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not an appropriate marker strain for fitness assays in complex environments such as plants, where pantothenate cannot be continuously supplied. A spontaneous streptomycin resistant strain (SBW25-Sm) has been used (Gal et al., 2003), but like other antibiotic resistance markers, the two competitor strains have to be enumerated separately in different agar plates supplemented with and without the antibiotic. Therefore, the systematic environmental errors associated with antibiotic markers are usually large and consequently, their ability to report small fitness effects is limited.

Here we describe the construction and validation of a neutral marker for *P. fluorescens* SBW25. The marked strain (SBW25-*lacZ*) was generated by integrating a promoterless '*lacZ*' gene into a defective prophage locus containing phage gene remnants in a 12.95 kb DNA region. The *lacZ* allele was derived from the *lacZY* reporter gene cassette in plasmid pUIC3 (Rainey, 1999). The *lacZ* gene product ( $\beta$ -galactosidase) catalyzes the hydrolysis of X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside), which produces a blue colour. It is important to note that the *lacY* allele was not included because it encodes a membrane protein (lactose permease), which can result in a decrease of fitness.

The putative phage region (2-kb) was cloned from SBW25 cells by a standard SOE-PCR protocol (SOE: splicing by overlapping extension, Horton et al., 1989). As shown in Fig. 1, two

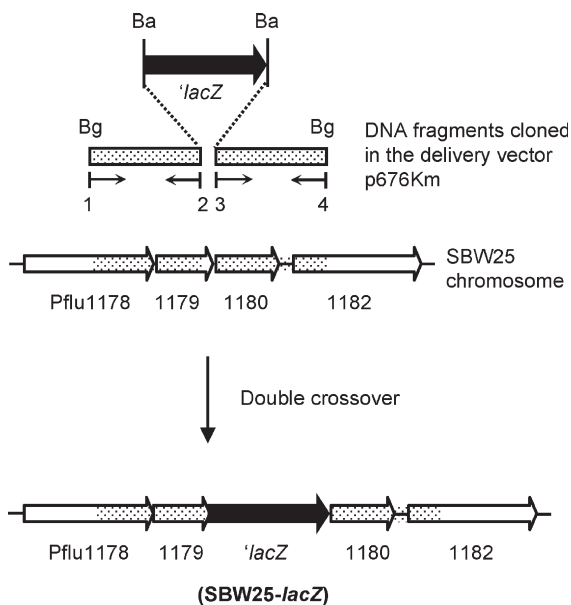


Fig. 1. Construction of neutrally marked strain *P. fluorescens* SBW25-*lacZ*. A 2-kb putative phage DNA (indicated by dotted bars) was amplified from the SBW25 genome by a standard procedure of SOE-PCR and subsequently cloned into vector pCR8 (Invitrogen). Next the 3-kb '*lacZ*' fragment was cloned into the middle of the phage region at the *Bam*HI site. The whole 5-kb insert was then retrieved by *Bgl*III digestion and cloned into the suicide delivery vector p676Km. The resulting delivery plasmid p676Km-*lacZ* was conjugated into *P. fluorescens* SBW25 and insertion of the '*lacZ*' by double crossover was achieved by a two-step allelic exchange strategy described in the text. The promoterless '*lacZ*' was inserted into the putative phage locus by replacing the 69 nucleotides of the *P. fluorescens* SBW25 genome (1312838 to 1312906). Locations of the oligonucleotide primers (Pha-1 to Pha-4) used to amplify the phage fragments are indicated by arrows with corresponding numbers 1 to 4. Restriction enzymes: Bg, *Bgl*III; Ba, *Bam*HI.

Table 1

Fitness of *P. fluorescens* SBW25-*lacZ* relative to the wild-type ancestor

Growth condition <sup>a</sup>	Selection rate constant (SRC) <sup>b</sup>
LB	0.060±0.045
King's B	0.077±0.073
M9 (with glucose and ammonia)	0.052±0.097
M9 salts plus histidine (15 mM)	0.076±0.044
M9 salts plus urocanate (15 mM)	0.072±0.066
PR ( <i>P<sub>i</sub></i> -rich medium)	-0.023±0.075
PM (medium with moderate <i>P<sub>i</sub></i> )	0.054±0.068
PL ( <i>P<sub>i</sub></i> -limited medium) <sup>c</sup>	-0.007±0.111
M9 plus bathocuproine disulfonate (100 μM)	0.109±0.061
M9 plus copper sulphate (1 μM)	0.080±0.089
LB 14 °C <sup>c</sup>	-0.100±0.134
Sugar beet (shoot)	-0.106±0.168
Sugar beet (rhizosphere)	-0.055±0.273

<sup>a</sup> *Pseudomonas* strains were grown at 28 °C unless specifically indicated. To test growth with various concentrations of inorganic phosphate (*P<sub>i</sub>*), a defined citrate medium (Al-Aoukaty et al., 1991) that contains citric acid (4.0 g/l), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1.0 g/l) and MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2 g/l) was used. Additionally, Na<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> were added at the concentration of 6.0 g/l and 3.0 g/l, respectively in *P<sub>i</sub>*-rich medium (PR), 3.0 g/l and 1.5 g/l, respectively in PM medium (with moderate *P<sub>i</sub>*), whereas in *P<sub>i</sub>*-limited (PL) medium they are added at 2.4 mg/l and 1.2 mg/l, respectively.

<sup>b</sup> Data are means and standard errors of 8–10 independent cultures. One sample *t*-tests show that the average values are not significantly different from zero (*P*<0.05).

<sup>c</sup> Fitness in PL (28 °C) and LB (14 °C) was performed for 2×48 hours with final cell density of 6.36±1.26×10<sup>6</sup> per ml and 5.57±0.59×10<sup>6</sup> per ml, respectively.

1-kb fragments corresponding to the DNA region spanning *pflu1178* to *pflu1182* were first amplified using two pairs of primers: Pha-1 (5'-GAGATCTCGCGCCATCGTCCCTGGC-3') and Pha-2 (5'-cagcatgcggatccgttgacggaGAGAATGTC-GACGCCGTTGA-3'); Pha-3 (tcgtaacggatccgcatgctgCCAAGGAGCTGCCCCATGGCCT-3') and Pha-4 (5'-GAGATCTGGGCGCCAGGGAATCGCTGT-3'). *Bgl*III or *Bam*HI restriction sites (italicized) were incorporated into the primers to facilitate subsequent cloning. Primers Pha-2 and Pha-3 contain additional complementary sequences (shown in small letters). This made it possible to join the two DNA fragments together via a PCR reaction using primers Pha-1/Pha-4. The resulting 2-kb PCR product was cloned into pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> (Invitrogen Auckland, NZ) to generate pCR8-1.

The '*lacZ*' gene was amplified from the DNA template of pUIC3 (Rainey, 1999) by PCR using primers lacZF-Bm (5-CGGGATCCTAATTAAGTAAGCTTGATATCGAAT-TCCCGG-3) and lacZR-Bm (5-CGGGATCCATAATG-GATTTCCTTACGC-3). To avoid the formation of a LacZ protein fusion that may greatly reduce the  $\beta$ -galactosidase activity, translational stop codons in all three reading frames (highlighted in bold) were incorporated into the 5' end of primer lacZF-Bm. The resulting 3-kb PCR product was cloned into pCR<sup>®</sup>8/GW/TOPO<sup>®</sup>, retrieved by *Bam*HI digestion and cloned into the *Bam*HI site of pCR8-1 to generate pCR8-2. The 5-kb *Bgl*III fragment (2-kb phage DNA with an insertion of the 3-kb '*lacZ*' in the middle, Fig. 1) from pCR8-2 was cloned into an integration vector, p676Km (Mob<sup>+</sup>, Km<sup>R</sup>, Zhang et al., 2006). The resulting plasmid, p676Km-*lacZ*, was used to deliver the '*lacZ*' marker gene into the genome of *P. fluorescens* SBW25.

Insertion of *lacZ* into the phage locus of SBW25 was achieved by a two-step allelic exchange strategy. p676Km-*lacZ* was mobilized into SBW25 by conjugation with the help of pRK2013 (Tra<sup>+</sup>, Km<sup>R</sup>). Integration by single homologous recombination was selected for on Luria-Bertani (LB, Sambrook et al., 1989) agar supplemented with nitrofurantoin (100 µg/ml to counterselect *E. coli*), Km (100 µg/ml), and X-gal (40 µg/ml). Then, to select for SBW25-*lacZ* strains with the plasmid vector excised, purified blue-coloured transconjugants were grown for two successive 24-hour periods in LB broth (600 ml). Bacterial cells were plated onto LB agar containing X-gal and colonies were screened for kanamycin resistance by replica-plating onto LB agar supplemented with Km (100 µg/ml). Integration of *lacZ* into the prophage locus in the blue and Km-sensitive colonies was confirmed by PCR amplification using primers Pha-1 and Pha-4: the desired mutants produced a 5-kb fragment, whereas the wild-type SBW25 produced a 2-kb fragment (data not shown). The verified *lacZ*-marked strain was designated SBW25-*lacZ*.

Performance of SBW25-*lacZ* growing in laboratory media and *in planta* was examined by direct competition with the wild-type ancestor. Inoculum cells were prepared as previously described (Zhang et al., 2006). Cells were first grown overnight in LB broth from cells stored at -80 °C (8–10 replicates) and then subcultured once in minimal M9 broth (Sambrook et al., 1989) for ~24 hours before they were co-cultured in the fitness test medium. This acclimation procedure ensured that the bacterial populations had comparable physiological states and cell densities prior to competition experiments. To initiate the competition, equal volumes (500 µl for each) of SBW25-*lacZ* and wild-type cells grown in M9 were mixed and then collected by centrifugation. Then the cell pellet was resuspended in 1 ml sterile water. The resulting bacterial suspensions were used to inoculate sugar beet seeds or laboratory media, and the actual initial frequencies of the competing strains were determined by dilution plating onto LB agar plates supplemented with X-gal.

For *in planta* fitness assays, mixed bacteria were inoculated onto coated sugar beet seeds (*Beta vulgaris* var. Amethyst. ~2000 cells per seed) as previously described (Zhang et al., 2006). The seeds were germinated and cultivated in 15 ml plastic tubes using non-sterile vermiculite as a growth substrate. After 14 days, cells in the phyllosphere and rhizosphere were harvested by removing the photosynthetic part of the seedling and roots with attached vermiculite, respectively and adding to 5 ml sterile water. After vortexing for 1 min at high speed, bacteria were enumerated on M9 agar plates supplemented with CFC (Cetrimide, Fucidin, and Cephalosporin from Oxoid Ltd, Hampshire, UK) and X-gal.

For fitness assays in laboratory media, approximately 10<sup>3</sup> mixed bacterial cells (5 µl) were inoculated into 5 ml test medium. After growth with shaking (150 rpm, 28 °C) for 24 hours, the cell density reached ~10<sup>9</sup> per ml in all media except for that which is specifically indicated in Table 1. Then the bacteria were then subcultured once in the same medium (5 µl into 5 ml). After two 24-hour transfers (~20 generations) in the laboratory media, the final bacterial densities were determined by enumerating single colonies on LB agar containing X-gal (40 µg/ml).

Population densities ( $N_i$ ) for both competitors determined at time  $t=0$  and at  $t=T$  were used to calculate the Malthusian parameter ( $m_i$ ) (Lenski et al., 1991), which is the average rate of increase:  $m_i = \ln[N_i(T)/N_i(0)]$ . Relative fitness is expressed here as the selection rate constant (SRC):  $r_{ij} = m_i - m_j$ , resulting in a fitness of zero when SBW25-*lacZ* and the unmodified SBW25 are equally fit.

Results are summarized in Table 1. The fitness of SBW25-*lacZ* is comparable with the wild-type ancestor when growing in routine laboratory media for the propagation of SBW25: LB and King's B media (King et al., 1954) and minimal medium M9 (Sambrook et al., 1989). No significant fitness alteration was observed when SBW25-*lacZ* was grown on histidine or its derivative (urocanate) as the sole carbon and nitrogen source; in minimal medium with various concentrations of phosphate; in M9 supplemented with bathocuproine disulfonate (BCS, a copper chelator, 100 µM) or copper sulphate (1 µM, the minimal growth inhibition concentration) (Table 1). The optimal growth temperature of SBW25 is 28 °C. SBW25-*lacZ* showed similar growth performance with the wild-type, when the growth temperature was reduced to 14 °C. Fitness assays in the plant environment showed that growth of SBW25-*lacZ* was not significantly different from the wild-type in both the shoot and the rhizosphere of sugar beet seedlings (Table 1), whereas fitness of the previous marked strain SBW25EeZY-6KX was significantly decreased relative to wild type (mean±standard error: -2.25±0.39 in the shoot and -2.24±0.35 in the rhizosphere). Additionally, Fukami et al. (2007) showed that SBW25-*lacZ* displayed similar diversification patterns compared to the wild-type ancestor when it was grown in 6 ml King's B broth under static conditions.

In summary, we have constructed a neutrally-marked strain (SBW25-*lacZ*) for the model organism *P. fluorescens* SBW25 and demonstrated its usefulness in relative fitness assays for a range of growing environments. The data show that fitness cost of the promoterless *lacZ* marker gene (excluding *lacY*) is negligible. The data also support our prediction that the prophage locus is a neutral site for genetic modification. Prophages are commonly found in bacterial genomes and many of them appear to be defective and are in a state of mutational decay (Casjens, 2003). Therefore, the same strategy reported here can be adopted for the construction of neutral markers in other bacteria.

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