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Note

Novel *tnpR*-based transposable promoter traps suitable for RIVET studies in different gram-negative bacteria

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ABSTRACT

The preparation of plasmid-borne RIVET libraries can be troublesome when high genomic coverages are needed. We present here the construction and functional validation of a new set of miniTn5 promoter traps to generate *tnpR*-based RIVET libraries. The ability to generate *tnpR* transcriptional fusions by transposition will significantly facilitate the setup of RIVET studies in those bacteria where Tn5 transposition is operative.

Despite the constant advances in microbial genomic technologies, much remains to be learned about the way in which microorganisms respond to their natural environments. Several methods including differential fluorescence induction (DFI) (Allaway et al., 2001) in depth cDNA sequencing (Schluter et al., 2010; Sharma et al., 2010) and different variants of *in vivo* expression technology (Mahan et al., 1993) including RIVET recombination-based *in vivo* expression technology (Osorio et al., 2005) have been used in the last decade to discover new genes induced in complex niches in plant and animal tissues (Rediers et al., 2005). The complexity of natural environments and the limiting amount of samples often hamper the application of proteomic and transcriptomic analyses. In such situations, RIVET has proved to be a valuable tool since, after *tnpR* induction, an irreversible and inheritable phenotypic change occurs in specific clones allowing for their selection at the end of the assay.

RIVET systems generally comprise two functional modules: a) a promoter-probe vector that carries a promoterless resolvase (*i.e.*, *tnpR* from the *Tn*-γδ) for the generation of genomic transcriptional fusions, and b) a reporter DNA module carrying a selection cassette that becomes permanently modified after a resolvase-mediated site-specific recombination (at *res* sequences for TnpR). Clones displaying *tnpR* inductions during the experiment can be recognized and collected at the end of the assay (a form of *system memory*). Both, negative- (Camilli and Mekalanos, 1995) and positive-selection modules (Casavant et al., 2002, 2003; Livny and Friedman, 2004; Lozano et al., 2011; Osorio et al., 2005; Saviola et al., 2003) for RIVET systems have been reported. For the promoter-probe module, different formats have also been used including narrow-(Castillo et al., 2008; Gao and Teplitski, 2008; Osorio et al., 2005) and broad-host-range plasmids (Ballering et al., 2009; Bron et al., 2004; Zhang and Cheng, 2006) to generate libraries of inserts transcriptionally fused to *tnpR*. Nevertheless, the use of such plasmids is often troublesome because of a high incidence of chimeric inserts. The construction of RIVET libraries is a time-consuming process requiring a high number of clones to assure the generation of transcriptional fusions that cover as many ORFs as possible along with their corresponding intergenic regions. Recently, a new transposon constructed to create transcriptional fusions to the gene of the Cre recombinase has been reported (Harrison et al., 2011) to skip the construction of RIVET plasmid libraries. Unfortunately, while *tnpR*-based RIVET screenings, including resolvable cassettes with different resolution efficiencies (Osorio et al., 2005), have been used in various bacterial species (Ballering et al., 2009; Castillo et al., 2008; Frank et al., 2012; Gao and Teplitski, 2008; Lombardo et al., 2007; Saviola et al., 2003; Tamir-Ariel et al., 2007; Zbell et al., 2008) the use of *Cre-lox* RIVET tools have been less explored and only in a few bacterial species (Altier and Suyemoto, 1999; Bachmann et al., 2008; Tuntufye et al., 2012).

We report here the construction and functional validation of a new set of miniTn5 promoter traps to generate *tnpR* based RIVET libraries. Tn5 transposons have previously been shown to transpose into a wide range of bacteria with little insertion-site preference (Reznikoff, 2008). The RIVET-transposons described here were constructed by modification of a recently published synthetic miniTn5 bordered by

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two optimized 19-bp mosaic sequences (ME), and using a hyperactive *tnpA* transposase (Martinez-Garcia et al., 2011). First, plasmid pBAM1 (Martinez-Garcia et al., 2011) was digested with the restriction enzymes *Bam*HI and *Xba*I, and the product ligated with a *Bgl*II-*Xba*I fragment from plasmid pRIVET-R (Lozano et al., 2011) that contained the *tnpR*-lacZ promoterless gen fusion (Fig. 1A, *cf.* the miniTn5-*tnpR-lacZ* in plasmid pMES1). Since in transposition experiments the integration of pMES1 might occur in *recA*$^+$–*lac*$^+$ bacteria, a Δ*lacZ* transposon was constructed by *Sal*I digestion of pMES1 and religation (Fig. 1B, *cf.* the miniTn5-*tnpR* in plasmid pMES2). Finally, the replication origin from plasmid p15A obtained as a *Xba*I-*Nhe*I fragment from plasmid pSM10 (Selbitschka et al., 1995), was cloned into the *Xba*I site of the miniTn5-*tnpR-lacZ*. The resulting miniTn5-*tnpR-lacZ-ori* (Fig. 1C, *cf.* the transposon in pMES3) was generated in order to facilitate the recovery of the host DNA bordering the Tn insertion by simple digestion of total DNA, self-ligation, and transformation into *Escherichia coli*.

To evaluate the transposition frequencies of the new transposons, the vectors pMES1 and pMES3 – carrying *miniTn5-tnpR-lacZ* and *miniTn5-tnpR-lacZ-ori*, respectively – were conjugated from *E. coli* SM10-λpir (donor) (Miller and Mekalanos, 1988) to *E. coli* HB101 (A. Pühler, Germany), *Sinorhizobium meliloti* 2011 (J. Dénarié., France), and to *Burkholderia tropica* Mto293 (Estrada-De Los Santos et al., 2001).

S. meliloti was grown in TY medium (Beringer, 1974) at 28 °C. *B. tropica* and *E. coli* were grown in LB medium (Bertani, 1951) at 28 °C and 37 °C, respectively. Antibiotics were added as required at the following concentrations: 400 μg/ml streptomycin (Str) for *S. meliloti* 2011; 100 μg/ml rifampicin (Rif) for *E. coli* HB101; and 10 μg/ml nalidixic acid (Nal) for *B. tropica* Mto293. Kanamycin (Km) 25/150 μg/ml was used to select for the presence of the miniTn5 in *E. coli* and *B. tropica*/*S. meliloti*, respectively. Bacterial matings were performed as previously described by Simon et al. (1983). Briefly, 0.7 ml of late exponential phase cultures from donor and recipient cells (obtained after 6–12 h cultivation of a 1:100 dilution of a saturated culture in fresh medium) were mixed, centrifuged for 8 min at 3000 ×g, resuspended in 50 μl of the same culture medium, and loaded onto a Millipore filter (0.2 μm pore size) that was placed on the surface of a TY/LB agar plate. After a 3–12 h incubation at 37/28 °C (for *E. coli*/*S. meliloti* and *B. tropica* respectively) the mix was resuspended in a liquid culture medium, and appropriate dilutions were plated on a selective medium with antibiotics to detect transposition. The resulting transposition frequencies per recipient bacteria and the proportions of vector integrations events were calculated, and are shown in Table 1. The transposition frequencies, which proved to depend upon the bacterial host used, resulted, in all instances, suitable for the construction of high-coverage RIVET libraries (depending on the type of bacterial host, several thousand transposants can be obtained by performing between 1 and 20 independent matings).

The suitability of the miniTn5-*tnpR-lacZ-ori* to be used in RIVET studies was demonstrated by the capacity to generate fusions that yielded an active *tnpR* that excised *res1* flanked DNA. To test the functionality of the mini-transposon as a promoter probe, we performed a generalized mutagenesis on strain *S. meliloti* 2011R1ΩT (Str^R, Tc^R), which strain we constructed by replacing the *nptII* by a ΩTc (Fellay et al., 1987) in the chromosome of the strain *S. meliloti* 2011R1ΩNGG (Lozano et al., 2011). The resolution of the *res1*-flanked Tc-resistance cassette in the transposants led to a Tc^S phenotype, and occurred at a frequency of 11.5 ± 2.7%. This frequency corresponds to the proportion of sense transcriptional fusions to *tnpR* whose promoter activities in strain *S. meliloti* 2011R1ΩT were enough to induce the excision of the *res1*-flanked Tc resistance.

The new miniTn5 RIVET tool constructed here was able to transpose in α-, β-, and γ-proteobacteria (Table 1.), and was found to express an active *tnpR*. The possibility of generating *tnpR* transcriptional fusions without the need of conventional plasmid-borne RIVET libraries will significantly facilitate the setup of RIVET studies in those bacteria where Tn5 transposition is operative. Interestingly, the RIVET clones generated by transposition corresponds to mutants affected at the site of the *tnpR* fusion, and will therefore also serve to investigate the phenotypic consequences of alterations within genes induced during the RIVET screening.

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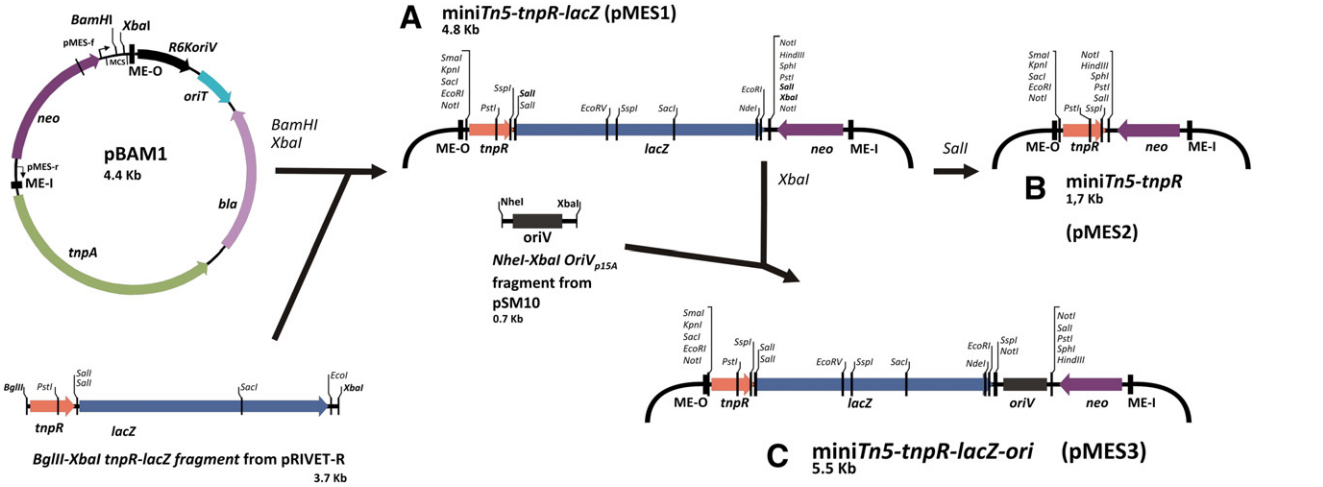

Fig. 1. Cloning strategy used for the construction of the new promoter-probe miniTn5 RIVET transposons. A. Plasmid pMES1 that carries the miniTn5-*tnpR-lacZ*; B. Plasmid pMES2 with the miniTn5-*tnpR* for the generation of RIVET libraries on *lac*$^+$rec$^+$ *E. coli* strains; and C. Plasmid pMES3 that carries the miniTn5-*tnpR-lacZ-ori* with the replication origin from plasmid p15A to facilitate cloning of the host-DNA bordering the transposon.

Table 1
Transposition frequencies of the new RIVET miniTn5 promoter probes.

RIVET miniTn5	Donor strain[a]	Recipient strain[a]	Transposition frequency (transposants/recipient)[b]	Integration(%)[c,d]
miniTn5-*tnpR-lacZ*	*E. coli* SM10-λpir	*E. coli* HB101	$1.49 \pm 0.34 \times 10^{-3}$	<1
miniTn5-*tnpR-lacZ*	*E. coli* SM10-λpir	*B. tropica* Mto293	$7.27 \pm 3.47 \times 10^{-5}$	8.7
miniTn5-*tnpR-lacZ-ori*	*E. coli* SM10-λpir	*S. meliloti* 2011	$4.18 \pm 2.96 \times 10^{-6}$	9.1

[a] For strain description see the references cited in the text.
[b] Mean ± Standard deviation. Mean values were calculated from three independent (n = 3) conjugation experiments (biological replicas). The transposition frequencies were calculated as the number of Km^R transconjugants per recipient, and corrected by the integration percentages in each instance.
[c] The integration frequencies in *E. coli* HB101 was calculated as the percentage of Amp^R bacteria with respect to the total number of transconjugants (Km^R–Rif^R clones).
[d] Integration frequencies in *S. meliloti* and *B. tropica* Mto293 were determined by PCR with the primers OriR6Ki-R (5′-CGTTACATCCCTGGCTTGTT-3′) and oriT-RP4-R (5′-AAAAGGTACCGCTTTTCCGCTGCATAAC-3′).

References

Allaway, D., Schofield, N.A., Leonard, M.E., Gilardoni, L., Finan, T.M., Poole, P.S., 2001. Use of differential fluorescence induction and optical trapping to isolate environmentally induced genes. Environ. Microbiol. 3, 397–406.

Altier, C., Suyemoto, M., 1999. A recombinase-based selection of differentially expressed bacterial genes. Gene 240, 99–106.

Bachmann, H., Kleerebezem, M., van Hylckama Vlieg, J.E., 2008. High-throughput identification and validation of in situ-expressed genes of *Lactococcus lactis*. Appl. Environ. Microbiol. 74, 4727–4736.

Ballering, K.S., Kristich, C.J., Grindle, S.M., Oromendia, A., Beattie, D.T., Dunny, G.M., 2009. Functional genomics of *Enterococcus faecalis*: multiple novel genetic determinants for biofilm formation in the core genome. J. Bacteriol. 191, 2806–2814.

Beringer, J.E., 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84, 188–198.

Bertani, G., 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. 62, 293–300.

Bron, P.A., Grangette, C., Mercenier, A., de Vos, W.M., Kleerebezem, M., 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J. Bacteriol. 186, 5721–5729.

Camilli, A., Mekalanos, J.J., 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. Mol. Microbiol. 18, 671–683.

Casavant, N.C., Beattie, G.A., Phillips, G.J., Halverson, L.J., 2002. Site-specific recombination-based genetic system for reporting transient or low-level gene expression. Appl. Environ. Microbiol. 68, 3588–3596.

Casavant, N.C., Thompson, D., Beattie, G.A., Phillips, G.J., Halverson, L.J., 2003. Use of a site-specific recombination-based biosensor for detecting bioavailable toluene and related compounds on roots. Environ. Microbiol. 5, 238–249.

Castillo, A.R., Woodruff, A.J., Connolly, L.E., Sause, W.E., Ottemann, K.M., 2008. Recombination-based *in vivo* expression technology identifies *Helicobacter pylori* genes important for host colonization. Infect. Immun. 76, 5632–5644.

Estrada-De Los Santos, P., Bustillos-Cristales, R., Caballero-Mellado, J., 2001. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl. Environ. Microbiol. 67, 2790–2798.

Fellay, R., Frey, J., Krisch, H., 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. Gene 52, 147–154.

Frank, K.L., Barnes, A.M.T., Grindle, S.M., Manias, D.A., Schlievert, P.M., Dunny, G.M., 2012. Use of recombinase-based *in vivo* expression technology to characterize *Enterococcus faecalis* gene expression during infection identifies *in vivo*-expressed antisense RNAs and implicates the protease Eep in pathogenesis. Infect. Immun. 80, 539–549.

Gao, M., Teplitski, M., 2008. RIVET-a tool for *in vivo* analysis of symbiotically relevant gene expression in *Sinorhizobium meliloti*. Mol. Plant Microbe Interact. 21, 162–170.

Harrison, C.L., Crook, M.B., Peco, G., Long, S.R., Griffitts, J.S., 2011. Employing site-specific recombination for conditional genetic analysis in *Sinorhizobium meliloti*. Appl. Environ. Microbiol. 77, 3916–3922.

Livny, J., Friedman, D.I., 2004. Characterizing spontaneous induction of Stx encoding phages using a selectable reporter system. Mol. Microbiol. 51, 1691–1704.

Lombardo, M.J., Michalski, J., Martinez-Wilson, H., Morin, C., Hilton, T., Osorio, C.G., Nataro, J.P., Tacket, C.O., Camilli, A., Kaper, J.B., 2007. An *in vivo* expression technology screen for *Vibrio cholerae* genes expressed in human volunteers. Proc. Natl. Acad. Sci. U. S. A. 104, 18229–18234.

Lozano, M.J., Salas, M.E., Giusti, M.A., Draghi, W.O., Torres Tejerizo, G.A., Martini, M.C., Del Papa, M.F., Pistorio, M., Lagares, A., 2011. Development of new positive-selection RIVET tools: detection of induced promoters by the excision-based transcriptional activation of an *aacCI* (Gm^R)–*gfp* fusion. J. Biotechnol. 155, 147–155.

Mahan, M.J., Slauch, J.M., Mekalanos, J.J., 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science 259, 686–688.

Martinez-Garcia, E., Calles, B., Arevalo-Rodriguez, M., de Lorenzo, V., 2011. pBAM1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes. BMC Microbiol. 11, 38.

Miller, V.L., Mekalanos, J.J., 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. J. Bacteriol. 170, 2575–2583.

Osorio, C.G., Crawford, J.A., Michalski, J., Martinez-Wilson, H., Kaper, J.B., Camilli, A., 2005. Second-generation recombination-based *in vivo* expression technology for large-scale screening for *Vibrio cholerae* genes induced during infection of the mouse small intestine. Infect. Immun. 73, 972–980.

Rediers, H., Rainey, P.B., Vanderleyden, J., De Mot, R., 2005. Unraveling the secret lives of bacteria: use of *in vivo* expression technology and differential fluorescence induction promoter traps as tools for exploring niche-specific gene expression. Microbiol. Mol. Biol. Rev. 69, 217–261.

Reznikoff, W.S., 2008. Transposon Tn5. Annu. Rev. Genet. 42, 269–286.

Saviola, B., Woolwine, S.C., Bishai, W.R., 2003. Isolation of acid-inducible genes of *Mycobacterium tuberculosis* with the use of recombinase-based *in vivo* expression technology. Infect. Immun. 71, 1379–1388.

Schluter, J.P., Reinkensmeier, J., Daschkey, S., Evguenieva-Hackenberg, E., Janssen, S., Janicke, S., Becker, J.D., Giegerich, R., Becker, A., 2010. A genome-wide survey of sRNAs in the symbiotic nitrogen-fixing alpha-proteobacterium *Sinorhizobium meliloti*. BMC Genomics 11, 245.

Selbitschka, W., Dresing, U., Hagen, M., Niemann, S., Pühler, A., 1995. A biological containment system for *Rhizobium meliloti* based on the use of recombination-deficient (*recA*-) strains. FEMS Microbiol. Ecol. 16, 223–232.

Sharma, C.M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., Chabas, S., Reiche, K., Hackermuller, J., Reinhardt, R., Stadler, P.F., Vogel, J., 2010. The primary transcriptome of the major human pathogen *Helicobacter pylori*. Nature 464, 250–255.

Simon, R., Priefer, U., Puhler, A., 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology (N. Y) 1, 784–791.

Tamir-Ariel, D., Navon, N., Burdman, S., 2007. Identification of genes in *Xanthomonas campestris* pv. vesicatoria induced during its interaction with tomato. J. Bacteriol. 189, 6359–6371.

Tuntufye, H.N., Lebeer, S., Gwakisa, P.S., Goddeeris, B.M., 2012. Identification of avian pathogenic *Escherichia coli* genes that are induced in vivo during infection in chickens. Appl. Environ. Microbiol. 78, 3343–3351.

Zbell, A.L., Maier, S.E., Maier, R.J., 2008. *Salmonella enterica* serovar Typhimurium NiFe uptake-type hydrogenases are differentially expressed in vivo. Infect. Immun. 76, 4445–4454.

Zhang, X.S., Cheng, H.P., 2006. Identification of *Sinorhizobium meliloti* early symbiotic genes by use of a positive functional screen. Appl. Environ. Microbiol. 72, 2738–2748.