Efficient transposon mutagenesis mediated by an IPTG-controlled conditional suicide plasmid

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Abstract

Background: Transposon mutagenesis is highly valuable for bacterial genetic and genomic studies. The transposons are usually delivered into host cells through conjugation or electroporation of a suicide plasmid. However, many bacterial species cannot be efficiently conjugated or transformed for transposon saturation mutagenesis. For this reason, temperature-sensitive (*ts*) plasmids have also been developed for transposon mutagenesis, but prolonged incubation at high temperatures to induce *ts* plasmid loss can be harmful to the hosts and lead to enrichment of mutants with adaptive genetic changes. In addition, the *ts* phenotype of a plasmid is often strain- or species-specific, as it may become non-*ts* or suicidal in different bacterial species.

Results: We have engineered several conditional suicide plasmids that have a broad host range and whose loss is IPTG-controlled. One construct, which has the highest stability in the absence of IPTG induction, was then used as a curable vector to deliver hyperactive miniTn5 transposons for insertional mutagenesis. Our analyses show that these new tools can be used for efficient and regulatable transposon mutagenesis in *Escherichia coli*, *Acinetobacter baylyi* and *Pseudomonas aeruginosa*. In *P. aeruginosa* PAO1, we have used this method to generate a Tn5 insertion library with an estimated diversity of ~10^8, which is ~2 logs larger than the best transposon insertional library of PAO1 and related *Pseudomonas* strains previously reported.

Conclusion: We have developed a number of IPTG-controlled conditional suicide plasmids. By exploiting one of them for transposon delivery, a highly efficient and broadly useful mutagenesis system has been developed. As the assay condition is mild, we believe that our methodology will have broad applications in microbiology research.

Background

Transposon mutagenesis is a powerful technique for bacterial genetic and genomic studies. One of the most widely used transposons is derived from Tn5. The Tn5 transposon contains two IS50 elements as inverted terminal repeats (Additional file 1: Figure S1) [1, 2]. Both IS50 and Tn5 can be mobilized by their encoded transposase (Tnp) protein, which recognizes two 19 base pair (bp) sequences at their ends, namely outside end (OE) and inside end (IE), for transposition [2]. OE and IE differ by 7 bp (Additional file 1: Figure S1). As Tn5 insertion is almost completely random, it can insert into any gene in a bacterium. The native Tn5/IS50 is not very active, thus avoiding overt deleterious effect on their hosts, but hyperactive mutants have been engineered as genetic manipulation tools [2, 3]. The most active one contains a mosaic sequence of OE and IE (mosaic end; ME) at the transposon termini and an engineered *tnp* gene encoding a highly active transposase enzyme (Tnp^H), which together increase Tn5 transposition by more than 1000-fold.

Transposons for insertional mutagenesis are usually delivered into bacteria through conjugation of a suicide plasmid [4-6]. Insertion mutants are then selected as the transposons are tagged with an antibiotic-resistance gene. The success of a transposon mutagenesis assay, especially a saturation mutagenesis assay, requires generation of an insertion library with high diversity, which requires efficient plasmid conjugation and transposon transposition. However, conjugation is inefficient in many bacterial species. Occasionally, electroporation has also been used to deliver transposon-containing suicide plasmids for mutagenesis, but low library diversities were often achieved using

such approaches [7-9]. To perform efficient transposon mutagenesis in these organisms, temperature-sensitive (*ts*) plasmids are sometimes used for transposon delivery [10-16]. However, many organisms do not have a *ts* and easily manipulatable plasmid, and sometimes a *ts* plasmid in one organism is either non-*ts* or suicidal in a different organism [10, 14, 17]. In addition, a high temperature is often required to cure the *ts* plasmids after mutagenesis, which can be inhibitory to cell growth and may result in selection of mutants with adaptive genetic changes [10, 11, 14].

In this study, we have developed an efficient and regulatable transposon mutagenesis tool that exploits an IPTG-controlled conditional suicide plasmid. It contains an RSF1010 replicon, an IncQ-type replication origin that allows plasmid replication in most Gram-negative bacteria, as well as a few Gram-positive bacteria [18]. It is relatively small, so it can be easily modified. To control plasmid replication by IPTG, a second copy of the plasmid-encoded *repF* repressor gene is cloned downstream of the *Escherichia coli tac* promoter. For efficient and regulatable transposon mutagenesis, we used miniTn5 (mTn5) transposons and cloned the hyperactive transposase gene downstream of a *lac* promoter. We show that the resulting constructs can be used for efficient insertional mutagenesis in three different bacterial species. In *Pseudomonas aeruginosa* PAO1, we show that our system is able to generate a Tn5 insertion library that is almost 2 logs larger than the best library of PAO1 and related *Pseudomonas* strains previously reported, demonstrating that we have developed a powerful mutagenesis tool that is highly useful for microbiology studies.

Results

Construction of IPTG-controlled suicide plasmids

To develop a method for efficient transposon mutagenesis in bacterial species that are difficult to transform and conjugate, we created multiple IPTG-controlled suicide plasmids that have a broad host range (Fig. 1a). The plasmids were derived from pMMB208, which is a conjugatable plasmid containing an RSF1010 *oriV* (an IncQ-type origin of replication) that can replicate in most Gram-negative bacteria and a few Gram-positive bacteria [18]. Plasmid replication requires three proteins, RepA, MobA/RepB and RepC, which are a helicase, a primase and an *oriV*-binding protein, respectively. *repF* encodes a small repressor protein that binds the P4 promoter and controls the *repF-repA-repC* operon through feedback inhibition [19, 20]. pMMB208 also contains a *tac* promoter (P*tac*), a *lacI*^{Q} gene and a chloramphenicol resistance marker (*Cam*^{R}). To create a conditional suicide plasmid (pMMB-*repF*), a second copy of the *repF* gene was inserted downstream of P*tac*. Upon IPTG induction, efficient plasmid loss from transformed *E. coli* DH10B cells was observed (99.97%; Fig. 1b). As an alternative strategy, we inserted two *repA* helicase dominant negative mutants, K42A and D139A, downstream of P*tac* [21]. Similarly, IPTG was able to induce efficient plasmid loss from the transformed DH10B cells. In fact, plasmid retention rates of the dominant negative mutants (K42A, 4.9×10^{-7}; D139A, 1.5×10^{-5}) were much lower than that of pMMB-*repF* (3.5×10^{-4}) (Fig. 1b). However, the two *repA* dominant negative mutants showed significantly lower plasmid stability in the absence of IPTG induction (Fig. 1b), suggesting that plasmid replication is strongly inhibited by leaky expression of the dominant negative mutants, or by spontaneous recombination of the wild-type and the dominant negative *repA* genes (~861 bp direct repeats). Consistent with that, there were ~20-30-fold less plasmid isolated from the same

amount of cells for the two mutant constructs (Fig. 1c). Therefore, we decided to choose pMMB-*repF* for further experiments. To kill the cells that still retain the plasmid after IPTG induction, we inserted a *sacB* counter selection marker into the vector [22], resulting in pMMB-*repF*/*sacB*. Indeed, insertion of *sacB* allows efficient killing of plasmid-containing cells by sucrose (data not shown; also see below).

IPTG-controlled mutagenesis of *E. coli* by a highly-active mTn5 transposon

A *Kan*^{R}-tagged mTn5 was then inserted in pMMB-*repF*/*sacB* for transposon mutagenesis (Fig. 2a) [4]. The mTn5 contains an OE and an IE at the termini. In addition, it contains an uncoupled, *lac* promoter (P*lac*)-controlled *tnp*^{H} gene encoding the hyperactive transposase (Tnp^{H}) [3], thus allowing inducible expression of Tnp^{H}. *E. coli* cells transformed with this plasmid, pSNC-mTn5, were cultured in LB media with and without IPTG induction for 24 h. Cells were then analyzed for efficiencies of plasmid loss, sucrose counter selection and transposon insertion (See Materials and Methods). The plasmid is stable without IPTG induction, as ~91.4% of cells retained the plasmid (Cam^{R}) after 24 h culture in the absence of antibiotics (Fig. 2b). In contrast, ~2.6 x 10^{-3} of the cells retained the plasmid post IPTG induction, suggesting that overexpression of the RepF repressor caused efficient plasmid loss. Sucrose counter selection further reduced plasmid-bearing cells (~1.6 x 10^{-6} are Cam^{R}; ~1600-fold reduction). In comparison, the percentage of Suc^{R}Kan^{R} cells after IPTG induction was found to be ~2.3 x 10^{-4} (Tn5-containing), significantly higher than that of Suc^{R}Cam^{R} cells (~1.6 x 10^{-6}, plasmid-containing), suggesting that Tn5 transposition had occurred efficiently (Suc^{R}Kan^{R}Cam^{S}: ~2.3 x 10^{-4}). Colony restreaking showed that 150/150 Suc^{R}Kan^{R} colonies were Kan^{R}Cam^{S} (Fig. 2c).

Colony PCRs, which used two sets of primers (P1+P2 for detection of *Kan*^{R}, or mTn5, and P3+P4 for detection of *tac-repF*, or plasmid), confirmed plasmid loss in 10 out of 10 colonies (10/10) (Fig. 2d). Sequence analysis showed that all 13 Suc^{R}Kan^{R} colonies analyzed had different Tn5 insertion sites (Fig. 3a).

Efficient mutagenesis of *Acinetobacter baylyi* and *P. aeruginosa* by a highly-active mTn5 transposon

Construct pSNC-mTn5 was then tested in two Gram-negative, capsule-bearing bacteria, *A. baylyi* 33305 and *P. aeruginosa* PAO1 [23, 24]. Comparing to *E. coli* DH10B, transformed *A. baylyi* 33305 and *P. aeruginosa* PAO1 appeared to lose the plasmid more easily in the absence of IPTG, with ~56.3% of *A. baylyi* and ~59.6% of *P. aeruginosa* retaining the plasmid after 24 h culture in LB media without antibiotics (Fig. 2e, f). Following IPTG induction, ~14.7% of *A. baylyi* and ~3.2% of *P. aeruginosa* retained the plasmid, suggesting that IPTG induced additional plasmid loss from these organisms, although their efficiencies were lower than that in DH10B cells. With sucrose counter selection, ~1.6 x 10^{-7} of *A. baylyi* remained Cam^{R}, indicating that they contained the plasmid (Fig. 2e). Similarly, ~5.4 x 10^{-7} of *P. aeruginosa* cells were found to be Suc^{R}Cam^{R} (Fig. 2f). These results suggest that IPTG and sucrose both contributed in reducing plasmid-bearing cells. In comparison, the percentages of Suc^{R}Kan^{R} cells were 8.4 x 10^{-6} for *A. baylyi* and 3.4 x 10^{-6} for *P. aeruginosa*, suggesting that Tn5 transposition occurred in both organisms prior to plasmid loss. Colony restreaking showed that 100/100 Suc^{R}Kan^{R} colonies are Suc^{R}Cam^{S}, suggesting that efficient plasmid loss had occurred following Tn5 transposition (~100% for both; Additional file 1: Figure S2a, c). Loss of

plasmids was further confirmed by PCR tests (Additional file 1: Figure S2b, d). As observed in DH10B cells, Tn5 insertion also seemed to be random, as 9/9 *A. baylyi* and 13/15 *P. aeruginosa* mutants had different Tn5 insertion sites (Fig. 3b, c). The detection of identical mutants suggests that cell growth ensued following transposon transposition (Fig. 3c), which is common in different transposon mutagenesis assays [5, 6, 25].

Construction of a Tn5 insertion library of *P. aeruginosa* using the highly-active mTn5 transposon

To determine whether we can construct a transposon insertion library of *P. aeruginosa* PAO1 with high diversity, ten pSNC-mTn5 transformants of the bacterium were cultured independently and then combined and induced with IPTG to initiate transposon mutagenesis. Following 24 h culture in LB media containing IPTG, ~6.4% of cells retained the plasmid (Additional file 1: Figure S3a). The frequencies of $Suc^{R}Cam^{R}$ and $Suc^{R}Kan^{R}$ cells in the IPTG-induced culture were found to be ~6.5 x 10^{-7} and ~3.5 x 10^{-6}, respectively. Based on the total number of cells cultured and the frequency of $Suc^{R}Kan^{R}Cam^{S}$ cells, the total diversity of the mTn5 insertion library was estimated to be ~1.3 x 10^{7}, which covers the entire gene repertoire (5697) of *P. aeruginosa* PAO1 by ~2,238 times [24]. To our knowledge, the diversity of this transposon insertion library is bigger than the best transposon insertion library of PAO1 and related strains previously reported (Table 1) [5, 6, 9, 26-32]. Colony restreaking and PCR tests confirmed plasmid loss in the mutants (Additional file 1: Figure S3b, c), and 28/37 clones analyzed had different Tn5 insertion sites (Additional file 1: Figure S3d). Based on the percentage of independent clones in the library, its diversity is re-estimated to be ~1.0 x 10^{7}**.**

Table 1 Comparison of transposon insertion libraries of *P. aeruginosa* strains

Pseudomonas strains	Total mutants	Transposon	Method	References
P. aeruginosa PAO1	**~75,000,000**	mTn5ME	IPTG-curable	This study
P. aeruginosa PAO1	**~10,000,000**	mTn5	IPTG-curable	This study
P. aeruginosa PAO1	~80,000	Himar1	Conjugation	[26]
P. aeruginosa PAO1	~18,500	Tn5	Conjugation	[27]
P. aeruginosa MPAO1[a]	~2,172,750[c]	Tn5	Conjugation	[6]
P. aeruginosa MPAO1	~100,000	Tn5	Conjugation	[28]
P. aeruginosa MPAO1	~45,409	Tn5	Conjugation	[5]
P. aeruginosa PAO1SR[b]	~1,000,000	Himar1	Conjugation	[29]
P. aeruginosa PA14	~300,000[d]	Himar1	Conjugation	[30]
P. aeruginosa PA14	~38,976	Himar1	Conjugation	[31]
P. aeruginosa QR1	~15,000	Himar1	Conjugation	[32]

[a] A PAO1 derivative with ~0.2% genetic changes
[b] A streptomycin resistant derivative of PAO1 with unknown changes
[c] Combination of 16 experiments
[d] Combination of 100 independent conjugations

An mTn5 with MEs enables generation of a *P. aeruginosa* mutant library with even higher diversity

To determine whether the efficiency of mTn5 transposition can be further improved, we replaced both OE and IE of the mTn5 with MEs (Fig. 4a). The new plasmid, pSNC-mTn5ME, was transformed into DH10B cells. Cell growth (or colony sizes) appeared to be normal, suggesting that basal-level transposition, if any, did not lead to obvious cellular toxicity, which was our initial concern. The behavior of the plasmid and Tn5 transposition efficiency were determined under the same conditions described above. Without IPTG induction, the plasmid remained relatively stable, as ~100% of the cells retained the plasmid (Cam^R). After IPTG induction for 24 h, 1.1 x 10^{-3} of the cells retained the plasmid, suggesting that RepF overexpression caused efficient plasmid loss. With sucrose counter selection, ~2.4 x 10^{-5} cells remained Suc^RCam^R. Interestingly, the frequency of Suc^RKan^R cells was found to be very high (~28.0%), indicating that mTn5ME is much more active

than the non-ME version (~1200 folds). Restreaking of $Suc^{R}Kan^{R}$ colonies showed that they were all $Kan^{R}Cam^{S}$ (100/100) (Additional file 1: Figure S4a), and colony PCRs confirmed plasmid loss (10/10) (Additional file 1: Figure S4b). Sequence analysis of the transposon insertion junctions showed that all 13 $Suc^{R}Kan^{R}$ colonies analyzed had different Tn5 integration sites (Additional file 1: Figure S4c).

We then determined whether construct pSNC-mTn5ME would also be more active in *A. baylyi* 33305 and in *P. aeruginosa* PAO1. For *A. baylyi*, the efficiencies of plasmid loss were higher for pSNC-mTn5ME than for pSNC-mTn5, both in the absence and presence of IPTG induction (Fig. 2e and 4c). Sucrose counter selection was highly effective for both constructs (Fig. 2e and 4c). As in *E. coli*, mTn5ME was found to be much more active than the non-ME version (~140 fold higher; $Suc^{R}Kan^{R}$ cells: ~1.2 x 10^{-3} for mTn5ME vs. ~8.4 x 10^{-6} for mTn5). Similarly, colony restreaking of $Suc^{R}Kan^{R}$ cells showed that 100/100 colonies are $Kan^{R}Cam^{S}$ (Additional file 1: Figure S5a), and plasmid loss was further verified by PCR (Additional file 1: Figure S5b). 9/14 colonies were found to have different Tn5 insertion sites (Additional file 1: Figure S5c). For PAO1, efficiencies of plasmid loss (±IPTG) were found to be similar for both pSNC-mTn5 and pSNC-mTn5ME (Fig. 2f and 4d), and sucrose counter selection was also effective for pSNC-mTn5ME (Fig. 4d). As in *E. coli* and in *A. baylyi*, mTn5ME was found to be more active than mTn5 in PAO1 (~11 fold higher; $Suc^{R}Kan^{R}$ cells: ~3.9 x 10^{-5} for mTn5ME vs. ~3.4 x 10^{-6} for mTn5) (Fig. 2f and 4d). In the colony restreaking assay, 100/100 $Suc^{R}Kan^{R}$ colonies were found to be $Kan^{R}Cam^{S}$ (Additional file 1: Figure S6a), and PCR assays further confirmed plasmid loss (10/10) (Additional file 1: Figure S6b). Sequence

232 determination showed that 7/10 colonies tested had different Tn5 insertion sites
233 (Additional file 1: Figure S6c).

234 We then determined whether pSNC-mTn5ME would be a better construct than
235 pSNC-mTn5 for transposon saturation mutagenesis in *P. aeruginosa*. Ten transformants
236 were randomly picked for Tn5 insertion library construction using the protocol described
237 above. About 0.87% of cells retained the plasmid after IPTG induction, and the frequency
238 of $Suc^{R}Cam^{R}$ cells was found to be 6.8×10^{-8}. In comparison, the frequency of $Suc^{R}Kan^{R}$
239 cells was found to be ~9.5×10^{-5}, suggesting that efficient mTn5ME transposition has
240 occurred. Based on the total amount of cells cultured and the mTn5ME transposition
241 efficiency (~9.5×10^{-5}), the diversity of the mTn5ME insertion library was estimated to be
242 1.02×10^{8}, which is ~3 logs larger than the best PAO1 transposon insertion library
243 previously reported and ~2 logs larger than a Tn5 insertion library of *P. aeruginosa*
244 MPAO1, a derivative of PAO1 with ~0.2% genetic variation [6, 33]. This new library is by
245 far the biggest transposon insertion library of PAO1 and related species ever reported
246 (Table 1). The size of our new library is enough to cover the entire gene repertoire of
247 PAO1 by ~18,000 times. Colony restreaking (100) and PCR tests (10) confirmed plasmid
248 loss in all the $Suc^{R}Kan^{R}$ clones analyzed (Fig. 4f, g), and 34/46 clones tested had different
249 Tn5 insertion sites (Fig. 4h). Thus, the independent clones in the library (library diversity)
250 are estimated to be ~7.5×10^{7}**.**
251

252 **Discussion**

253 We have developed a new transposon mutagenesis system that is efficient, regulatable,
254 easy-to-use, and broadly useful. We believe it will be especially useful for functional

genomics studies of Gram-negative bacteria that are difficult to transform and conjugate, such as certain capsule-containing bacteria, obligate anaerobes, and possibly obligate intracellular pathogens as well. The advantage of this method relies on the following features: (i) A broadly-functional plasmid replicon; (ii) Replication of the plasmid is regulated by IPTG; (iii) The inclusion of the *sacB* gene for counter selection; (iv) A highly-active/hyperactive transposon; (v) Regulatable expression of the hyperactive transposase gene; (vi) mTn5 and mTn5ME transposons insert almost completely randomly in different bacteria (Additional file 1: Figure S7) [34, 35]. In addition, the relatively small sizes of the RSF1010-based plasmids also facilitate their transformation and conjugation. Similar to transposon mutagenesis using *ts* plasmids, our system does not depend on efficient plasmid transformation and conjugation, and requires as few as one transformant or conjugated cell for transposon saturation mutagenesis. Using this new tool, we have generated a Tn5 transposon insertion library of *P. aeruginosa* PAO1 with a diversity of ~10^8, which is ~2 logs larger than the best transposon insertion library of PAO1 and related *Pseudomonas* strains ever generated (Table 1). *P. aeruginosa* is an important opportunistic pathogen that frequently causes nosocomial infections and many of the strains are multidrug-resistant. The mutant PAO1 library we generated should also be valuable for *P. aeruginosa* pathogenesis studies.

To our knowledge, our plasmids are the only non-*ts*, conditional suicide plasmids used for transposon mutagenesis, and they replicate in a wide range of bacterial species [19]. In contrast, many *ts* mutant plasmids seem to have limited host ranges, either due to the limited host ranges of the parental plasmids, or due to the species-specificity of their *ts* phenotypes [10, 11, 14-17, 36]. In addition, *ts* plasmids often require prolonged

278 incubation at high temperatures for plasmid curing, which can be harsh conditions for
279 bacterial growth and survival, thus may lead to accumulation of adaptive genetic changes.
280 Table S1 in Additional file 1 is a detailed comparison of our systems (pSNC-mTn5 and
281 pSNC-mTn5ME) with various *ts* plasmid-based platforms that have been used for
282 transposon mutagenesis in Gram-negative bacterial species, which clearly shows that
283 our systems will be more broadly useful. In addition to their utilities in transposon
284 mutagenesis, the IPTG-controlled conditional suicide plasmids that we developed should
285 have many other applications, such as for allelic exchange or as curable vectors for
286 delivering gene targeting systems, *e.g.*, TargeTrons, λ Red, RecET, *etc* [37, 38].
287

288 **Conclusion**

289 In this work, we have developed a number of IPTG-controlled conditional suicide plasmids
290 that contain the broad-host-range RSF1010 origin. Using one of the constructs to deliver
291 a hyperactive mTn5 transposon, we showed that this system can be used for efficient
292 mutagenesis of different bacterial species. As the assay condition is mild and the host
293 range of the RSF1010 plasmid is extremely wide, we believe that our methodology will
294 have broad applications in microbiology research.
295

296 **Methods**

297 **Bacterial strains and growth conditions**

298 *E. coli* DH10B was purchased from Invitrogen. *A. baylyi* (ATCC 33305) and *P. aeruginosa*
299 PAO1 (ATCC BAA-47) were purchased from ATCC. Unless stated otherwise, all the
300 strains were grown at 37°C in Luria Broth (LB) liquid media with agitation at 200 rpm or

on LB plates with 1.5% agar. For *sacB* counter selection, we used LBNS plates (LB no salt: 1% Tryptone, 0.5% yeast extract and 1.5% agar) supplemented with 10% sucrose (Fisher Scientific). Appropriate antibiotics and concentrations were used to select for bacterial cells that are antibiotic resistant. *E. coli* DH10B: chloramphenicol (Cam; Gold Biotechnology), 25 µg/ml; kanamycin (Kan; Fisher Scientific), 50 µg/ml. *A. baylyi*: Cam, 10 µg/ml; Kan, 10 µg/ml. *P. aeruginosa* PAO1: Cam, 250 µg/ml; Kan, 500 µg/ml.

Plasmid construction

To construct plamsid pMMB-*repF*, we PCR-amplified the *repF* gene from pMMB208 [18]. The PCR fragment was digested with *Hin*dIII and *Pst*I, and inserted at the corresponding sites of pMMB208, downstream of the *tac* promoter (P*tac*). To construct plasmid pMMB-*repF*/*sacB*, the *sacB* gene and its promoter were PCR amplified from plasmid pRE112 [22] and inserted between the unique *Sac*I and *Kpn*I sites of pMMB-*repF*.

To construct plasmids pMMB-*repA*K42A and pMMB-*repA*D139A, *repA* genes containing K42A and D139A mutations were generated in two-step PCRs from plasmid pMMB208 [21]. The mutant genes were cloned between the *Hin*dIII and *Pst*I sites of pMMB208.

Plasmid pSNC-mTn5 was constructed in multiple steps. First, plasmid pUT-mTn5Km/lacEZ was constructed from plasmid pUT-mTn5Km [4]. It contains a *lac* promoter-driven hyperactive transposase gene (tnp^H) that has E54K, M56A and L372P mutations [3]. In addition, inside the mTn5 transposon, the inverted repeats flanking the kanamycin resistance marker (Kan^R) were deleted [4]. The entire mTn5 cassette of pUT-mTn5Km/lacEZ, which contains the Kan^R-mTn5 transposon and P*lac*-tnp^H, was then PCR

amplified and cloned at the *Xba*I site of pMMB-*repF*/*sacB*, resulting in plasmid pSNC-mTn5. It has an OE and an IE at the termini of the mTn5. Plasmid pSNC-mTn5ME was derived from pSNC-mTn5 by replacing both OE and IE with MEs.

Characterization of IPTG-induced plasmid loss and Transposon mutagenesis

To test IPTG-induced plasmid loss of pMMB-*repF*, pMMB-*repA*K42A and pMMB-*repA*D139A, single colonies of *E. coli* DH10B cells transformed with the plasmids were inoculated into 5 ml LB+Cam media and cultured at 37°C for ~14 hours (h). After measuring OD_{600}, 1 ml of each culture was pelleted by centrifugation and washed with 500 µl of fresh LB to remove antibiotics. Cells were then resuspended in 1 ml LB. An aliquot was added to 5 ml LB (final OD_{600} = 0.001) with and without 1 mM IPTG and cultured at 37°C for 24 h. 1 ml of the IPTG-induced samples was then pelleted, washed with 500 µl LB, and resuspended in 1 ml LB. Serial dilutions of the samples (±IPTG) were plated on LB and LB+Cam plates to evaluate plasmid loss. Plasmid retention frequencies were calculated as ratios of cfu (colony forming units) on LB+Cam plates and those on LB plates.

To perform transposon mutagenesis in *E. coli* DH10B, single colonies of pSNC-mTn5 and pSNC-mTn5ME transformants were cultured in 5 ml LB+Cam+Kan media overnight at 37°C. Cells were then pelleted and washed as above to remove antibiotics, and an aliquot was inoculated to 5 ml LB (final OD_{600} = 0.001) in a 14 ml culture tube and grown at 37°C for 24 h with and without 1 mM IPTG induction. A 1 ml aliquot of the IPTG-induced samples was then pelleted, washed with 500 µl LBNS, and resuspended in 1 ml LBNS. Serial dilutions of the samples (±IPTG) were plated on LB and LB+Cam plates to

evaluate plasmid loss. The IPTG induced samples were also plated on LBNS+10% sucrose and LBNS+10% sucrose+Cam plates to estimate percentage of plasmid-retaining cells in the presence of sucrose counter selection; and LBNS+10% sucrose+Kan plates to select for transposition events. Plasmid retention frequencies (PRF) were calculated as the following: (1) -IPTG: (cfu on LB+Cam)/(cfu on LB); (2) +IPTG: (cfu on LB+Cam)/(cfu on LB); (3) +IPTG+Suc: (cfu on LBNS+Suc+Cam)/(cfu on LBNS+Suc). Transposon retention frequencies (TRF) were calculated as the following: +IPTG+Suc: (cfu on LBNS+Suc+Kan)/(cfu on LBNS+Suc). mTn5 (or mTn5ME) transposition frequencies were calculated as $TRF_{+IPTG+Suc} - PRF_{+IPTG+Suc}$, which essentially equals to $TRF_{+IPTG+Suc}$ if the background ($PRF_{+IPTG+Suc}$) is low. The same protocol, except for the concentrations of antibiotics (indicated above) and IPTG (10 mM for PAO1), was followed to perform transposon mutagenesis in *P. aeruginosa* PAO1.

Similarly, to perform transposon mutagenesis in *A. baylyi* 33305, single colonies of pSNC-mTn5 and pSNC-mTn5ME transformants were cultured in 5 ml LB+Cam+Kan media overnight at 37°C. Cells were pelleted and washed as for *E. coli* and *P. aeruginosa*. Then, an aliquot was inoculated to 100 ml LB (final OD_{600} = 0.001) in a baffled flask. The cultures were shaken vigorously (~250 rpm) at 37°C for 24 h with and without 10 mM IPTG induction. A 1 ml aliquot of the IPTG-induced samples was then pelleted, washed with 500 µl LBNS, and resuspended in 1 ml LBNS. Serial dilutions of the samples (±IPTG) were then plated on appropriate plates to evaluate plasmid loss and mTn5 (or mTn5ME) transposition as in the assays for *E. coli* and for *P. aeruginosa*.

To verify plasmid loss in cells with potential transposition events, 100-150 $Suc^{R}Kan^{R}$ colonies in each assay were then restreaked on LB+Kan and LB+Cam plates.

In addition, presence of the transposon and the plasmid was determined by colony PCRs in a 25 µl reaction containing 25 mM TAPS-HCl (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1x GC enhancer, 0.2 mM dNTPs, 0.1 µl of Q5 polymerase (2 u/µl; NEB), 1 µl of resuspended cells, and 150 ng each of the primers (final concentration = ~0.5 µM; see figure legends and Table S2 in Additional file 1 for oligos used). PCRs were performed using the following condition: 1x (94°C, 2 minutes); 25x (94°C, 30 seconds; 50°C, 30 seconds; 72°C, 1 minute); 1x (72°C, 10 minutes); 1x (4°C, hold).

Determination of transposon insertion sites

Transposon insertion sites in bacterial chromosomes were determined by arbitrarily primed PCR, in which transposon junctions were amplified in two steps [5, 39]. Bacterial cells were resuspended in 10-20 µl of deionized water and 1 µl was used directly as the PCR template. In the first PCR step, the reaction was performed using a specific primer annealing to the transposon region (Tn5Km1) and a semi-degenerate primer (BDC1) that anneals to many sites on the bacterial chromosome. In the second step, aliquots of the first-round PCR products were amplified using a primer annealing to the transposon region (Tn5Km2), slightly closer to the insertion junction, and a non-degenerate primer (BDC2) that anneals to the constant region of the BDC1-derived sequence. PCRs were carried out under the conditions described above. PCR products from Step 2 were resolved in a 2% agarose gel and major products were gel-purified for sequencing to determine Tn5 insertion sites.

Construction of transposon insertion libraries of *P. aeruginosa* PAO1

To construct an mTn5 (or mTn5ME) insertion library of *P. aeruginosa* PAO1, plasmid pSNC-mTn5 (or pSNC-mTn5ME) was first electroporated into the bacterial cells. Ten transformants were cultured independently in 5 ml LB+Cam+Kan media at 37°C for ~14 hours. Equal amount of each sample (equivalent to 0.5 OD_{600} x 1 ml) was then combined, pelleted, washed with 500 µl LB, and the pellet was resuspended in 1 ml LB. An aliquot of the mixture was then inoculated into 500 ml LB supplemented with 10 mM IPTG in a baffled flask (final OD_{600} = 0.01) and shaken vigorously (300 rpm) at 37°C for 24 h to perform transposon mutagenesis. The cells were then pelleted by centrifugation and washed with 250 ml LBNS medium. The pellet was resuspended in 50 ml LBNS medium and serial dilutions were plated on LB, LB+Cam, LBNS+10% sucrose, LBNS+10% sucrose+Cam, and LBNS+10% sucrose+Kan plates to determine plasmid loss, mTn5 (mTn5ME) transposition, and total library diversity. Arbitrary PCR and DNA sequencing were then performed to determine Tn5 insertion sites.

Determination of mTn5 and mTn5ME target site preferences in *P. aeruginosa* PAO1

To determine if mTn5 and mTn5ME have any target site preferences in *P. aeruginosa* PAO1, we generated sequence logos of their insertion sites in the bacterium using the WebLogo server (https://weblogo.berkeley.edu/logo.cgi). In total, 40 mTn5 insertion sites and 41 mTn5ME insertion sites were used for the analysis.

Abbreviations

IPTG: Isopropyl β-D-1-thiogalactopyranoside; *ts*: Temperature-Sensitive; bp: base pair; OE: Outside End; IE: Inside End; ME: Mosaic End; OD: Optical Density; LB: Luria Broth; LBNS: LB no salt; Cam: Chloramphenicol; Kan: kanamycin.

Acknowledgements

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Availability of data and materials

The dataset supporting the conclusion of this article are available from the corresponding author on reasonable request.

Authors' contributions

SSN, HG conceived the study and designed the experiments; SSN, JH, SYZ, JZ, AS, LBG, CVS, FR, performed the experiments; SSN and HG, wrote the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interests.

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553

554

Figure Legends

Fig. 1 IPTG-controlled conditional suicide plasmids. (**a**) Plasmid pMMB208 and its conditional-suicide derivatives. pMMB208 contains an RSF1010 *oriV* for replication and an *oriT* for conjugation. Genes *repA*, *mobA*/*repB* and *repC* encode proteins required for plasmid replication, and *repF* encodes a transcription repressor that binds promoter P4. pMMB208 also has Cam^R and $lacI^Q$ genes and a P*tac* promoter. Plasmid pMMB-*repF* is a derivative of pMMB208 that has a second copy of the *repF* gene inserted downstream of P*tac*. Plasmids pMMB-*repA*K42A and pMMB-*repA*D139A have a dominant-negative *repA* mutant gene, either K42A or D139A, inserted downstream of P*tac*. (**b**) Amount of *E. coli* DH10B cells retaining the indicated plasmids after 24 h growth in the absence of antibiotics, either with or without IPTG induction. Results were average of three independent experiments, and bars represent mean ± SD (standard deviation). *p < 0.0001, **p < 0.0001, and ***p < 0.0001 by unpaired Student's t-test for IPTG induced cultures. (**c**) pMMB208 and its derivatives are digested with *Hin*dIII (H) and *Pst*I (P). Comparing to pMMB208 and pMMB-*repF*, the *repA* K42A and D139A mutants showed reduced yields in plasmid minipreps (no IPTG induction; 3.0% and 4.7% of that of pMMB208, respectively). *Hin*dIII and *Pst*I digestion generates two fragments for each plasmid. The ~9 kb fragment is seen on the gel, while the shorter ones, ranging from 18 bp for pMMB208 to 861 bp for the *repA* mutants, are not visible. Another large band (~9 kb) is also seen in restriction digestion of pMMB208 and its derivatives, even after complete digestion, and the cause is unknown.

Fig. 2 mTn5 transposon mutagenesis using an IPTG-controlled conditional suicide plasmid. (**a**) Diagram of plasmid pSNC-mTn5. pSNC-mTn5 is a derivative of *pMMB-repF* that contains a *Kan*^{R}-tagged mTn5, a *lac* promoter-controlled hyperactive transposase gene (*tnp*^{H}), and a *sacB* counter selection marker (with its own promoter). OE and IE are outside and inside ends of the mTn5. (**b**) Plasmid and transposon retention frequencies in *E. coli* DH10B. A "+" symbol for IPTG indicates that the inducer was added to the liquid culture, and a "+" symbol for Suc, Cam, and Kan indicates that the chemicals were added to the plates. Black columns represent plasmid retention frequencies, and the blue column represents Tn5 retention frequency. Results were average of three independent experiments, and bars represent mean ± SD (*p < 0.0001 and **p = 0.0054 by unpaired t-test). (see Materials and Methods for details) (**c**) Colony restreaking. 150/150 Suc^{R}Kan^R colonies of DH10B were found to be Kan^{R}Cam^S and 50 are shown here. (**d**) Colony PCR of 10 restreaked clones in (**c**). Primer sets P1&P2 and P3&P4 detect *Kan*^{R} and *repF*, respectively. All were mTn5-positive and plasmid-negative. Primers P3 and P4 are a functional pair for PCR-amplification of the plasmid sequence (data not shown). (**e**) Plasmid and transposon retention frequencies in *A. baylyi.* Results were average of three independent experiments, and bars represent mean ± SD (*p = 0.024 and **p < 0.0001 by unpaired t-test). (**f**) Plasmid and transposon retention frequencies in *P. aeruginosa*. Results were average of three independent experiments, and bars represent mean ± SD (*p = 0.0013 and **p = 0.0038 by unpaired t-test). Colony restreaking and PCR analysis are shown in Additional file 1: Figure S2.

599 **Fig. 3** mTn5 insertion sites in different bacteria. (**a**) mTn5 insertion sites in *E. coli* DH10B.
600 (**b**) mTn5 insertion sites in *A. baylyi* 33305. (**c**) mTn5 insertion sites in *P. aeruginosa*
601 PAO1. Only the chromosomal sequences next to the OE are shown. The 9 bp duplicated
602 sequences are shown in capital letters. Identical clones are shown only once, with
603 numbers indicated in parenthesis. Either gene names or locus tags are given as genetic
604 locations.
605
606 **Fig. 4** Generation of a *P. aeruginosa* insertion library with pSNC-mTn5ME. (**a**) Diagram
607 of pSNC-mTn5ME, a derivative of pSNC-mTn5 that has MEs instead of OE and IE at the
608 termini of mTn5. (**b**) Plasmid and transposon retention frequencies in *E. coli* DH10B.
609 Results were average of three independent experiments, and bars represent mean ± SD
610 (*$p < 0.0001$ and **$p = 0.0004$ by unpaired t-test). Colony restreaking and PCR assays
611 are shown in Additional file 1: Figure S4. (**c**) Plasmid and transposon retention
612 frequencies in *A. baylyi*. Results were average of three independent experiments, and
613 bars represent mean ± SD (*$p = 0.0029$ and **$p = 0.0006$ by unpaired t-test). Colony
614 restreaking and PCR assays are shown in Additional file 1: Figure S5. (**d**) Plasmid and
615 transposon retention frequencies in *P. aeruginosa* PAO1. Results were average of three
616 independent experiments, and bars represent mean ± SD (*$p < 0.0001$ and **$p = 0.0065$
617 by unpaired t-test). Colony restreaking and PCR assays are shown in Additional file 1:
618 Figure S6. (**e**) Plasmid and transposon retention frequencies in the *P. aeruginosa* PAO1
619 mutant library generated with pSNC-mTn5ME. (**f**) Colony restreaking. 100/100 $Suc^{R}Kan^{R}$
620 colonies of the mTn5ME library of *P. aeruginosa* were found to be $Kan^{R}Cam^{S}$. 50 are
621 shown here. (**g**) Colony PCR of ten restreaked clones in (**f**) with the indicated primers. All

were mTn5-positive and plasmid-negative. (**h**) Transposon insertion sites of 46 mutant clones from the mTn5ME insertion library of *P. aeruginosa*. Identical clones are shown only once, with their duplication numbers indicated in parenthesis.

Additional files

Additional file 1: Figure S1. Tn5 transposons. (**a**) Full-length Tn5. The full-length Tn5 contains two inverted IS50 elements at its ends. Only one of them encodes an active Tnp and an Inh (Inhibitor of Tnp). *Kan*^{R}, kanamycin-resistance gene; *Str*^{R}, streptomycin-resistance gene; and *Ble*^{R}, bleomycin-resistance gene. (**b**) mTn5s. Top, an mTn5 with an OE and an IE at the termini. Bottom, an mTn5 with MEs at the ends. (**c**) Comparison of OE, IE and ME, with their polymorphisms highlighted in red.

Additional file 1: Figure S2. Confirmation of mTn5 transposition events in *A. baylyi* and in *P. aeruginosa*. (**a**) Colony restreaking assay of *A. baylyi*. 100 Suc^{R}Kan^R colonies of *A. baylyi* were restreaked on LB+Kan and LB+Cam plates, and all were found to be Kan^{R}Cam^S. 50 are shown here. (**b**) Colony PCR of 10 restreaked *A. baylyi* clones with the indicated primers. All were mTn5-positive and plasmid-negative. (**c**) Colony restreaking assay of *P. aeruginosa*. 100 Suc^{R}Kan^R colonies of *P. aeruginosa* were restreaked on LB+Kan and LB+Cam plates, and all were found to be Kan^{R}Cam^S. 50 are shown here. (**d**) Colony PCR of ten restreaked *P. aeruginosa* clones with primers indicated in the diagram. All were mTn5-positive and plasmid-negative.

644 **Additional file 1: Figure S3.** A transposon insertion library of *P. aeruginosa* PAO1
645 generated with pSNC-mTn5. (**a**) Plasmid and transposon retention frequencies of the
646 mTn5 insertion library of *P. aeruginosa* PAO1. (**b**) Colony restreaking assay. 100
647 random $Suc^{R}Kan^{R}$ colonies were restreaked on LB+Kan and LB+Cam plates. 100/100
648 were found to be $Kan^{R}Cam^{S}$ and 50 are shown here. (**c**) Colony PCR of ten restreaked
649 clones in (**b**). All were found to be mTn5-positive and plasmid-negative. (**d**) mTn5
650 insertion sites of 37 mutant clones from the transposon insertion library of *P.*
651 *aeruginosa*. Identical clones are only shown once, and their numbers are indicated in
652 parenthesis.
653
654 **Additional file 1: Figure S4.** Confirmation of mTn5ME transposition events in *E. coli*
655 DH10B. (**a**) Colony restreaking. 100 random $Suc^{R}Kan^{R}$ colonies of *E. coli* were
656 restreaked on LB+Kan and LB+Cam plates. 100/100 were found to be $Kan^{R}Cam^{S}$ and
657 50 restreaked colonies are shown here. (**b**) Colony PCR of ten restreaked clones in (**a**).
658 All were found to be Tn5-positive and plasmid-negative. (**c**) Tn5 insertion sites of 13
659 independent DH10B clones.
660
661 **Additional file 1: Figure S5.** Confirmation of mTn5ME transposition events in *A. baylyi*
662 33305. (**a**) Colony restreaking. 100 random $Suc^{R}Kan^{R}$ colonies of *A. baylyi* were
663 restreaked on LB+Kan and LB+Cam plates. 100/100 were found to be $Kan^{R}Cam^{S}$ and
664 50 restreaked colonies are shown here. (**b**) Colony PCR of ten restreaked clones in (**a**).
665 All were found to be Tn5-positive and plasmid-negative. (**c**) Sequence analysis shows
666 that 9/14 *A. baylyi* clones had different Tn5 insertion sites.

667

668 **Additional file 1: Figure S6.** Confirmation of mTn5ME transposition events in *P.*
669 *aeruginosa* PAO1. (**a**) Colony restreaking. 100 random $Suc^{R}Kan^{R}$ colonies of *P.*
670 *aeruginosa* PAO1 were restreaked on LB+Kan and LB+Cam plates. 100/100 were
671 found to be $Kan^{R}Cam^{S}$ and 50 restreaked colonies are shown here. (**b**) Colony PCR of
672 ten restreaked clones in (**a**). All were found to be Tn5-positive and plasmid-negative. (**c**)
673 Sequence analysis shows that 7/10 *P. aeruginosa* clones had different Tn5 insertion
674 sites.

675

676 **Additional file 1: Figure S7.** Target site preferences of mTn5 and mTn5ME in *P.*
677 *aeruginosa*. (**a**) Sequence logo of mTn5 insertion sites generated with WebLogo. 40
678 target sequences were analyzed. The 9 bp duplicated sequences adjacent to the OE
679 are shown. There is a slight preference for certain nucleotides at several positions. (**b**)
680 Sequence logo of mTn5ME insertion sites. 41 target sequences were analyzed. The 9
681 bp duplicated sequences adjacent to an ME are shown. It appears that mTn5ME has
682 less nucleotide preference at the duplicated target sequence than mTn5 in
683 *Pseudomonas*.

684

685 **Additional file 1: Table S1.** Comparison of conditional suicide vector-based transposon
686 mutagenesis strategies used in Gram-negative bacteria.

687

688 **Additional file 1: Table S2.** List of primers used.

Fig. 1

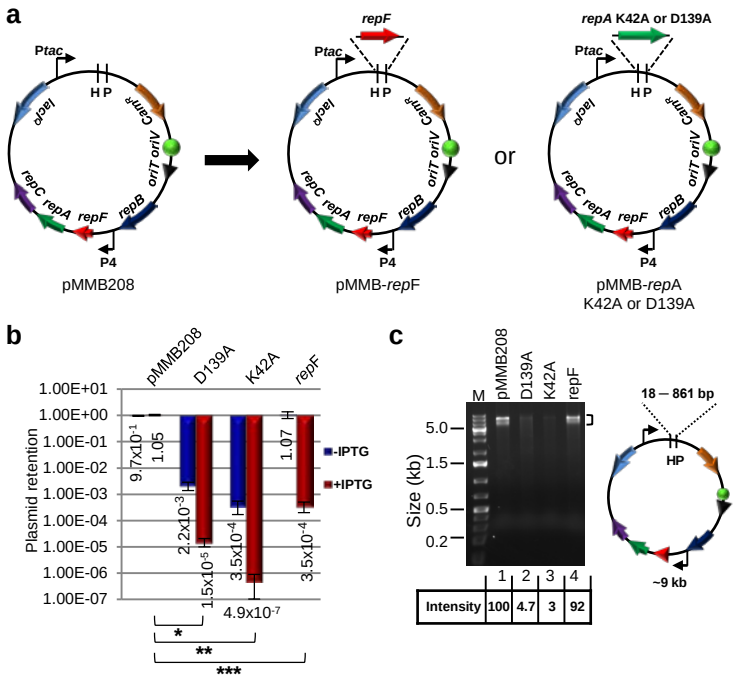

a
Ptac
H P
lacI^q
Cam^R
oriV
oriT
repB
repF
repA
repC
P4
pMMB208
repF
pMMB-repF
or
repA K42A or D139A
pMMB-repA
K42A or D139A
b
pMMB208
D139A
K42A
repF
Plasmid retention
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
9.7x10-1
1.05
2.2x10-3
1.5x10-5
3.5x10-4
4.9x10-7
1.07
3.5x10-4
-IPTG
+IPTG
*
**
***
c
M
pMMB208
D139A
K42A
repF
Size (kb)
5.0
1.5
0.5
0.2
1
2
3
4
Intensity
100
4.7
3
92
18 — 861 bp
HP
~9 kb

Fig. 2

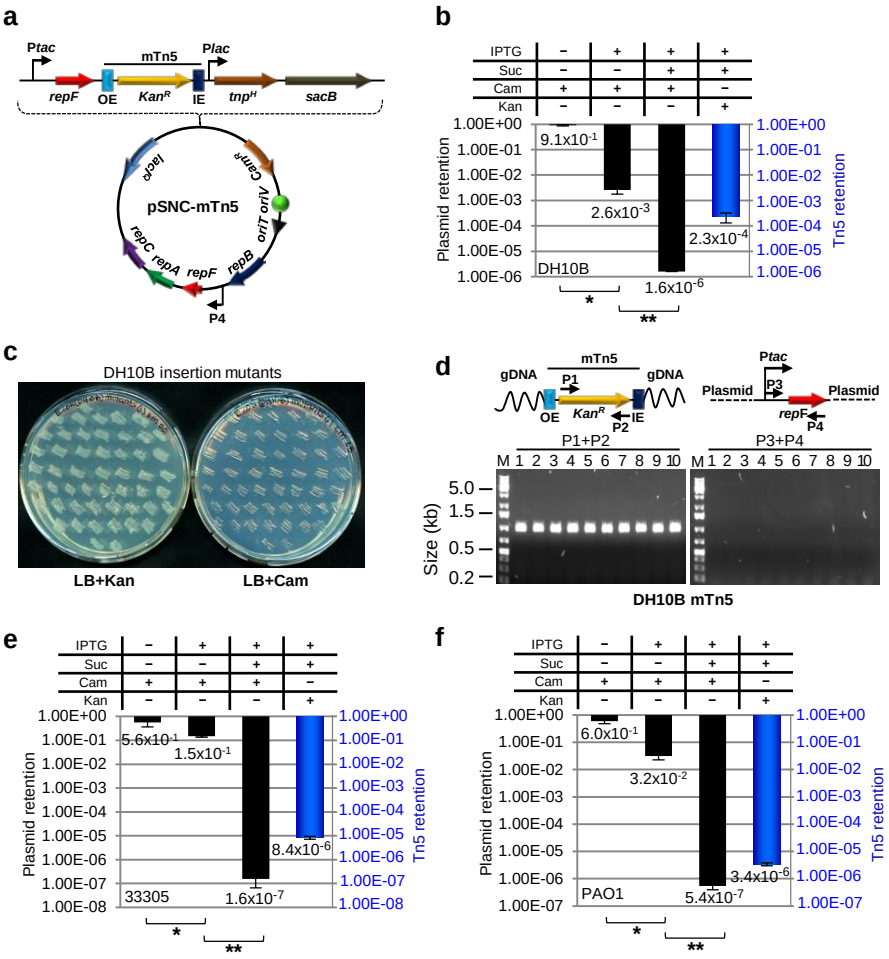

a
Ptac
mTn5
Plac
repF
OE
KanR
IE
tnpH
sacB
pSNC-mTn5
lacIq
CamR
oriV
oriT
repB
repF
repA
repC
P4
b
IPTG
Suc
Cam
Kan
Plasmid retention
Tn5 retention
9.1x10-1
2.6x10-3
2.3x10-4
1.6x10-6
DH10B
c
DH10B insertion mutants
LB+Kan
LB+Cam
d
gDNA
mTn5
P1
OE
KanR
IE
P2
Plasmid
P3
repF
P4
P1+P2
P3+P4
M 1 2 3 4 5 6 7 8 9 10
Size (kb)
5.0
1.5
0.5
0.2
DH10B mTn5
e
5.6x10-1
1.5x10-1
8.4x10-6
1.6x10-7
33305
f
6.0x10-1
3.2x10-2
3.4x10-6
5.4x10-7
PAO1

Fig. 3

a

E. coli DH10B

Clone	Target sequence<mTn5>	Genetic location
E1	acttatgGATTGCACG<OE-*kan*^{R}-IE>	*ygeV*
E2	tttgctgGGCAAAGGC<OE-*kan*^{R}-IE>	*mtr*
E3	tggcaacAGCCTGAAC<OE-*kan*^{R}-IE>	*crcA*
E4	ccacgcaGATTATGCC<OE-*kan*^{R}-IE>	*insF-1* [a]
E5	cgccaccGTGTTAGGG<OE-*kan*^{R}-IE>	Intergenic
E6	tttggtaCGTATGTAC<OE-*kan*^{R}-IE>	*yeaS*
E7	cttatcaGGCCTACGC<OE-*kan*^{R}-IE>	Intergenic[b]
E8	gcaattaGCCCGGGTC<OE-*kan*^{R}-IE>	*eda*
E9	cgcggcgGTGCTGGGC<OE-*kan*^{R}-IE>	*ltaE*
E10	gcttcaaGGATAGAGC<OE-*kan*^{R}-IE>	*yagL*
E11	tagggtaGAGACGCAG<OE-*kan*^{R}-IE>	*yahB*
E12	ctttacaCCTTAAGGC<OE-*kan*^{R}-IE>	Intergenic
E13	tcagtccTCTTTCACC<OE-*kan*^{R}-IE>	*yjhS*

[a] One of the five probable target sites
[b] One of the seven probable target sites

b

A. baylyi 33305

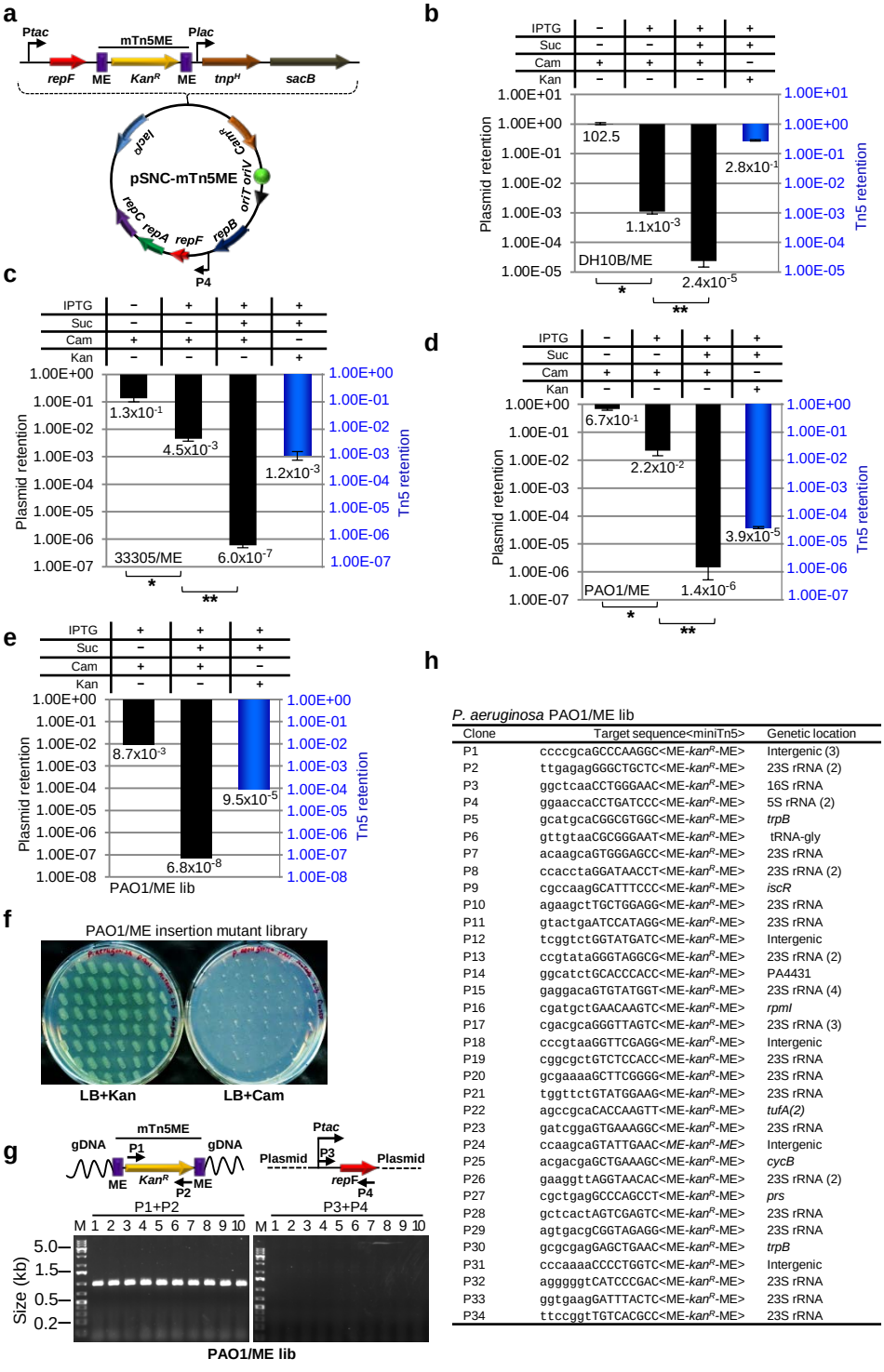
Clone	Target sequence<mTn5>	Genetic location
A1	aggtgtaGCGGTGAAA<OE-*kan*^{R}-IE>	16S rRNA
A2	tcactctGGTATAAAG<OE-*kan*^{R}-IE>	ACIAD1176
A3	gcgtaatATTAAAGGC<OE-*kan*^{R}-IE>	*UvrB*
A4	gtctacaGATCAAGCT<OE-*kan*^{R}-IE>	*pilU*
A5	cattgaaAGACTTAAG<OE-*kan*^{R}-IE>	ACIAD2819
A6	ttttactGTATTGGAC<OE-*kan*^{R}-IE>	ACIAD0088
A7	ggacattACCGAAGCC<OE-*kan*^{R}-IE>	ACIAD0046
A8	tggagcaACTTGATGC<OE-*kan*^{R}-IE>	*pilI*
A9	tatcaaaGTCTAAAGC<OE-*kan*^{R}-IE>	ACIAD3001

c

P. aeruginosa PAO1

Clone	Target sequence<mTn5>	Genetic location
P1	ttacgcaCCGCAAAGG<OE-*kan*^{R}-IE>	Intergenic
P2	tcgacaaCGATAAGGT<OE-*kan*^{R}-IE>	*flgM*
P3	ccagaagGCGGGCAGC<OE-*kan*^{R}-IE>	Intergenic(2)
P4	tctagtgGCCTAGGAC<OE-*kan*^{R}-IE>	tRNA-Glu
P5	tgaccaaGGTCTGCTC<OE-*kan*^{R}-IE>	*tufa*
P7	tcccccaGGGCGGACG<OE-*kan*^{R}-IE>	PA3806
P8	agccgcaCACCAAGTT<OE-*kan*^{R}-IE>	*tufa*
P9	tccaacgCCATGGGCC<OE-*kan*^{R}-IE>	*trpB*
P10	taggataGGTGGGAGG<OE-*kan*^{R}-IE>	23S rRNA
P11	ccccgcaGCCCAAGGC<OE-*kan*^{R}-IE>	Intergenic(2)
P13	gggggatCTTCGGACC<OE-*kan*^{R}-IE>	16S rRNA

Fig. 4



h

P. aeruginosa PAO1/ME lib

Clone	Target sequence<miniTn5>	Genetic location
P1	ccccgcaGCCCAAGGC<ME-*kan*^{R}-ME>	Intergenic (3)
P2	ttgagagGGGCTGCTC<ME-*kan*^{R}-ME>	23S rRNA (2)
P3	ggctcaaCCTGGGAAC<ME-*kan*^{R}-ME>	16S rRNA
P4	ggaaccaCCTGATCCC<ME-*kan*^{R}-ME>	5S rRNA (2)
P5	gcatgcaCGGCGTGGC<ME-*kan*^{R}-ME>	*trpB*
P6	gttgtaaCGCGGGAAT<ME-*kan*^{R}-ME>	tRNA-gly
P7	acaagcaGTGGGAGCC<ME-*kan*^{R}-ME>	23S rRNA
P8	ccacctaGGATAACCT<ME-*kan*^{R}-ME>	23S rRNA (2)
P9	cgccaagGCATTTCCC<ME-*kan*^{R}-ME>	*iscR*
P10	agaagctTGCTGGAGG<ME-*kan*^{R}-ME>	23S rRNA
P11	gtactgaATCCATAGG<ME-*kan*^{R}-ME>	23S rRNA
P12	tcggtctGGTATGATC<ME-*kan*^{R}-ME>	Intergenic
P13	ccgtataGGGTAGGCG<ME-*kan*^{R}-ME>	23S rRNA (2)
P14	ggcatctGCACCCACC<ME-*kan*^{R}-ME>	PA4431
P15	gaggacaGTGTATGGT<ME-*kan*^{R}-ME>	23S rRNA (4)
P16	cgatgctGAACAAGTC<ME-*kan*^{R}-ME>	*rpmI*
P17	cgacgcaGGGTTAGTC<ME-*kan*^{R}-ME>	23S rRNA (3)
P18	cccgtaaGGTTCGAGG<ME-*kan*^{R}-ME>	Intergenic
P19	cggcgctGTCTCCACC<ME-*kan*^{R}-ME>	23S rRNA
P20	gcgaaaaGCTTCGGGG<ME-*kan*^{R}-ME>	23S rRNA
P21	tggttctGTATGGAAG<ME-*kan*^{R}-ME>	23S rRNA
P22	agccgcaCACCAAGTT<ME-*kan*^{R}-ME>	*tufA(2)*
P23	gatcggaGTGAAAGGC<ME-*kan*^{R}-ME>	23S rRNA
P24	ccaagcaGTATTGAAC<*ME-kan*^{R}-*ME*>	Intergenic
P25	acgacgaGCTGAAAGC<ME-*kan*^{R}-ME>	*cycB*
P26	gaaggttAGGTAACAC<ME-*kan*^{R}-ME>	23S rRNA (2)
P27	cgctgagGCCCAGCCT<ME-*kan*^{R}-ME>	*prs*
P28	gctcactAGTCGAGTC<ME-*kan*^{R}-ME>	23S rRNA
P29	agtgacgCGGTAGAGG<ME-*kan*^{R}-ME>	23S rRNA
P30	gcgcgagGAGCTGAAC<ME-*kan*^{R}-ME>	*trpB*
P31	cccaaaaCCCCTGGTC<ME-*kan*^{R}-ME>	Intergenic
P32	agggggtCATCCCGAC<ME-*kan*^{R}-ME>	23S rRNA
P33	ggtgaagGATTTACTC<ME-*kan*^{R}-ME>	23S rRNA
P34	ttccggtTGTCACGCC<ME-*kan*^{R}-ME>	23S rRNA