Refactoring the conjugation machinery of promiscuous plasmid RP4 into a device for conversion of Gram-negative isolates to Hfr strains

by

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**SUPPLEMENTAL METHODS**

**Strains and media.** The strains of *S. cerevisiae, P. putida and E. coli* employed in this work are listed in Supplementary Table S1. *S. cerevisiae* was routinely grown at 30 °C/shaking (170 rpm) in liquid YPD (20 g L\(^{-1}\) Peptone, 10 g L\(^{-1}\) Yeast Extract and 20 g L\(^{-1}\) dextrose) or SC-Ura (6.7 g L\(^{-1}\) Yeast Nitrogen Base w/o amino acids, 1.92 g L\(^{-1}\) Yeast Synthetic Drop-out medium Supplement without uracil and 20 g L\(^{-1}\) dextrose). For agar plates, 2% w/v of agar was added to liquid media and autoclaved. Yeast stocks were made in liquid YPD with 15% glycerol and stored at -80 °C. *E. coli* and *P. putida* strains were grown in liquid LB with shaking (170 rpm) at 37 °C and 30 °C, respectively. M9 minimal media was prepared according to and supplemented with 0.2% (w/v) citrate or glucose as carbon sources and 20 µg ml\(^{-1}\) of Uracil (Ura) when stated. 2.5 ml of Goodies Solution (300 mg L\(^{-1}\) HBO\(_3\), 50 mg L\(^{-1}\) ZnCl\(_2\), 30 mg L\(^{-1}\) MnCl\(_2\)•4H\(_2\)O, 200 mg L\(^{-1}\) CoCl\(_2\), 10 mg L\(^{-1}\) CuCl\(_2\)•2H\(_2\)O, 20 mg L\(^{-1}\) NiCl\(_2\)•6H\(_2\)O, 30 mg L\(^{-1}\) NaMoO\(_4\)•2H\(_2\)O, 2.5 g L\(^{-1}\) FeSO\(_4\)•7H\(_2\)O, and 61.62 g L\(^{-1}\) MgSO\(_4\)•7H\(_2\)O) were added per liter of M9-Citrate agar media when necessary. Antibiotics were included in the growth media at the following concentrations: 150 µg ml\(^{-1}\) of ampicillin (Ap), 50 µg ml\(^{-1}\) of kanamycin (Km), 30 µg ml\(^{-1}\) of chloramphenicol (Cm), 100 µg ml\(^{-1}\) of streptomycin (Sm), 100 µg ml\(^{-1}\) of rifampicin (Rif) and 50 µg ml\(^{-1}\) of nalidixic acid (Nal). Gentamicin (Gm) was added at 10 µg ml\(^{-1}\) for *E. coli* and 15 µg ml\(^{-1}\) for *P. putida* growth in liquid media. Gm 50 µg ml\(^{-1}\) was used for selection of *P. putida* trans-conjugants in solid media during genome transfer experiments.

**General procedures and primers.** DNA manipulations were carried out following standard protocols \(^1\) and according to manufacturer recommendations. Primers used in this study are listed in Table S3 and were purchased from Sigma-Aldrich (St. Louis, USA). Standard plasmid purifications were performed with the QIAprep\textsuperscript{®} Spin Miniprep Kit (Qiagen, Valencia, USA). In the case of RP4, plasmid isolation kits worked very poorly for this large plasmid. Therefore, DNeasy\textsuperscript{®} UltraClean\textsuperscript{®} Microbial Kit (Qiagen) was used to isolate the whole DNA content of *E. coli* cells hosting RP4 and DNA samples were used as plasmid template for PCR reactions. In all cases, plasmid samples used as PCR templates were isolated from 5 ml culture and diluted 1:100 prior to amplification. DNA amplifications aimed for plasmid construction were performed with Q5 polymerase (New England Biolabs, Ipswich, USA). Diagnosis PCRs were conducted with DNA Amplitools Master Mix (Biotools, Madrid, Spain) and amplicon purifications were done with the
Nucleospin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). Localization of mini-Tn5 insertions was performed via the arbitrary PCR amplification protocol outlined by 2. DNA sequencing was outsourced to Macrogen (Spain). Special protocols were used for plasmid purification and colony PCR in S. cerevisiae (see “Yeast Assembly Methods” below). Transformation of E. coli laboratory strains was carried out with chemically competent cells using the CaCl₂ method 1. P. putida was transformed with plasmids via tripartite mating as described in 3 and selected in solid M9 minimal media supplemented with 0.2% w/v citrate and appropriate antibiotics. Other mating protocols used in this work are explained elsewhere in the Experimental Procedures Section.

Construction of P. putida strains for Genome transfer assays. Donor strains P. putida-TRANS#X are derivatives of P. putida EM42 4 with the TRANS device inserted in the genome via a mini-Tn5 transposon. They also display three mutations conferring Sm, Rif and Nal resistance and were constructed as follows: P. putida EM42 harboring the plasmid pSEVA2514-rec2 was first subjected to one cycle of recombineering with mutagenic oligos SR, RR and NR using the procedure outlined in 5. Mutations introduced by these oligos in target genes rpsL (PP_0449), rpoB (PP_0447) and gyrA (PP_1767) are translated to resistance to Sm, Rif, and Nal, respectively (see Table S3 for details). After selection on LB-agar plates supplemented with the three antibiotics, triple resistant colonies were streaked in the same media and the target genes were amplified with oligo pairs rpsL-Fw/rpsL-Rv, rpoB-F/rpoB-R, and gyrA-F/gyrA-R. The correct sequence changes were verified by sequencing the amplicons. One selected clone was cured of pSEVA2514-rec2 by iterative cultivation in LB without Km, obtaining the strain P. putida TA280. In order to insert the TRANS device into this strain, a bi-parental mating was performed as follows: P. putida TA280 and E. coli TransforMax™ EC100D™ pir⁺ (pTRANS) were inoculated, respectively, in 3 ml LB-Sm and 3 ml LB-ApKm. After overnight growth, cultures were adjusted to OD₆₀₀ ~1.0 and 1 ml of each was centrifuged 1 min/11000 rpm and resuspended in 1 ml of 10 mM MgSO₄. Aliquots of 200 µl were mixed in an Eppendorf tube, 1 ml of 10 mM MgSO₄ was added, and the aliquots were then spun down as before and resuspended in 10 µl of the same solution. This sample was placed on top of a LB-agar plate and incubated 18 h at 30 °C. The cellular patch was scraped out with an inoculation loop and resuspended in 1 ml of 10 mM MgSO₄. The bacterial suspension was plated in M9-Citrate-Km agar plates to select the P. putida TA280 trans-conjugants with mini-Tn5 insertions in
the genome. After overnight incubation at 30 ºC, around 200 colonies were obtained. Mini-Tn5 localization of 32 clones was performed as described in 2 by two rounds of arbitrary PCRs with oligo pairs MEO-Km-extF/ARB6 and MEO-Km-intF/ARB2. Eighteen representative clones of P. putida-TRANS#X with insertions throughout the genome were selected (Fig. 3) and two of them (#9 and #18) were assayed in this work.

The recipient strain was constructed by tagging P. putida EM42 ΔpyrFΔedd 8 with msfGFP via a modification of the delivery system described by 7. Briefly: a tetra-parental mating was set up with receptor P. putida EM42 ΔpyrFΔedd, donor E. coli (pTn7-M-PEM7-GFP) and the helper strains E. coli HB101 (pRK600) -for conjugation- and E. coli (pTNS2) -for transposition. The four strains were grown overnight in 3 ml LB supplemented with appropriate antibiotics (Table S1) and mated applying the protocol explained in the previous paragraph. Selection was made in M9-Citrate-Gm-Ura solid media and after incubation at 30 ºC for 18 hours, colonies showed green fluorescence. Some of them were streaked in the same media and subjected to diagnostic PCRs (primer pairs PS2/PP5408-F and PEM7-F/Tn7-GlmS) to confirm the correct integration of the transposon in the attTn7 locus. PCR analysis yielded the expected bands of 2.2 Kb and 1.2 Kb, respectively (data not shown). The double deletion ΔpyrF Δedd makes the obtained P. putida JS40 both uracil auxotrophic and unable to grow on media utilizing glucose as the sole carbon source, while the Tn7 insertion confers constitutive fluorescence and resistance to Gm. To properly check such a phenotype, this strain and donor P. putida-TRANS#9 were grown in different culture media (Fig. S2).

**Yeast assembly methods and plasmid construction.** The procedure for assembling plasmid constructs via homologous recombination in the yeast Saccharomyces cerevisiae followed the pipeline described in 8 with several modifications. In short, PCR amplicons were designed as contiguous DNA fragments with overlapping regions. The overlaps ranged from 40 nucleotides to 0.8 Kbp in length depending on the construct and were generated [i] as complementary tails included in the PCR primers [ii] as part of the PCR amplicons for adjacent sequences of a long DNA unit or [iii] as PCR linkers produced ad-hoc for non-adjacent fragments (see details below). DNA fragments designed for a given plasmid construct were PCR amplified and the products were purified and quantified. Equimolar mixtures of PCR fragments were used for yeast transformation.
Yeast were transformed via a modified version of the lithium acetate protocol: 2 mL YPD were inoculated with *S. cerevisiae* CRY1-2 and incubated overnight at 30 ºC with shaking. The next day, 25 ml of fresh YPD were inoculated with 1 ml of the overnight culture and grown at 30 ºC/170 rpm. Culture was monitored until OD$_{600}$ reached ~1.0 (around 5 hours). The cells were then spun at 3000 rpm for 5 minutes, then washed with 20 ml water, centrifuged in the same conditions and washed again with 20 ml of freshly made TE/LiOAc solution (100 mM lithium acetate at pH 7.5, 10 mM Tris-HCl, 1 mM EDTA). After centrifugation, cells were resuspended in 0.25 ml TE/LiOAc solution. 50 µl were moved to a new Eppendorf tube and spun briefly (3000 rpm/10 seconds) before removing the supernatant. The following solutions were added in the subsequent order: 240 µl 50% PEG-3350, 72 µl TE/LiOAc, 5 µl of denatured Herring sperm DNA (Promega D1811, 10 mg/ml; sample denatured upon arrival at 95 ºC/5 min, then incubated 10 min in ice, aliquoted and stored at -20 ºC until use) and the desired amount of DNA to be transformed. When necessary, the DNA sample pool was concentrated in a SpeedVac. The final volume was adjusted to 360 µl by addition of water and the transformation mixture was carefully resuspended by pipetting up and down several times. The mixture was incubated at 30 ºC for 30 minutes with gentle agitation every 10 minutes to avoid cell sedimentation. Then, heat shock at 42 ºC was applied for another 30 minutes with gentle shaking every 10 min. Afterwards, the cells were spun for 10 seconds at 4000 rpm and resuspended in 1 ml TE solution (10 mM Tris-HCl at pH 7.5, 1 mM EDTA) before being plated onto SC-Ura selection plates. Approximately 48 hours of incubation time at 30 ºC was necessary for appropriate growth of colonies. Colonies were then streaked onto SC-Ura plates and yeast colony PCRs were conducted to confirm successful transformations. For that purpose, each colony was resuspended in 50 µl of Solution 1 (Zymoprep™ Yeast Plasmid Miniprep II kit- ZymoResearch, Irvine, USA) and zymolase (REF #E1004, ZymoResearch, Irvine, USA) was added to a final concentration of 20 U/ml. The mixture was then incubated at room temperature for 10 minutes before centrifugation (10 seconds/4000 rpm) and elimination of the supernatant. The cell pellet was heated at 95 ºC for 5 minutes and then resuspended in 50 µl H$_2$O. Five µl of the suspension were used as template in a standard PCR reaction of 25 µl total volume. After gel visualization, a yeast miniprep was conducted for positive clones of *S. cerevisiae*. Yeast streaks were inoculated in 2 ml of SC-Ura liquid media and grown overnight. The protocol outlined in the Zymoprep™ Yeast Plasmid Miniprep II kit (ZymoResearch, Irvine, USA) was applied to 200 µl of the overnight cultures to isolate plasmid DNA. After elution
step, plasmid samples were evaporated in SpeedVac to concentrate them into a final volume of 10 µl. Five µL of this plasmid sample were then transformed into E. coli competent cells. Cells were plated in LB-Agar supplemented with appropriate antibiotics and resulting colonies were checked by miniprep, restriction analysis and sequencing (details of individual assemblies can be found in the following sections). More detailed information on buffer/reagents preparation can be found in 8.

**Assembly of pSEVA222Sβ (GenBank MW119277).** pSEVA222Sβ is a canonical SEVA plasmid featuring a RK2 origin of replication, a KmR cassette, a cargo lacZα-pUC19/I-SceI and a new gadget (β) which allows replication and selection in S. cerevisiae cells. The first three elements were PCR amplified from SEVA plasmids (see below) and the β gadget was recruited from plasmid pRS416. It contains the ARS209 origin of replication, the CEN6 centromeric sequence and the URA gene. A minimized version of each single element was designed to comply with the SEVA rules 10, removing non-functional DNA stretches from the original template. By the same token, the design removed prohibited restriction sites SwaI and PstI from the cognate sequence of CEN6 and URA, respectively. After amplification, purification and quantification of PCR-A (lacZα-pUC19/I-SceI from pSEVA212S), PCR-B (CEN6/ARS209 from pRS416, SwaI curated), PCR-C (partial sequence of URA3 from pRS416), PCR-D (partial sequence of URA3 from pRS416), PCR-E (sewing PCR of PCR-C+PCR-D to amplify the complete URA3 gene cured of a PstI site) and PCR-F (ori RK2 and KmR cassette from pSEVA2214), ~ 20 femtomols of the fragments PCR-A, -B, -E and -F were mixed and transformed into S. cerevisiae CRY1-2 following the procedure described above. In order to allow the assembly in S. cerevisiae via homologous recombination, amplification primers were added with sequence tails aimed to generate homologous regions of 40 nt between adjacent fragments. Details of PCR amplifications, templates, oligonucleotides and size/ functions of each amplified unit are depicted in Tables S2 and S3. After selection and re-streaking on SC-Ura agar plates, plasmid DNA from two selected S. cerevisiae clones was purified and electroporated into E. coli CC118 competent cells. Samples were plated on LB-Km agar and one colony from each transformation was first analyzed by miniprep and restriction analysis, then fully sequenced to ensure the accuracy of the construct. pSEVA222Sβ can replicate both in S. cerevisiae and E. coli. Replication in P. putida KT2440 was also verified (data not shown). Being endowed with a RK2 broad-host-range origin of replication, pSEVA222Sβ can potentially act as a
shuttle vector between any Gram- bacteria and *S. cerevisiae* and work as a Yeast Capture Vector for recruitment of large genomic fragments.

**Assembly of pTRANS (GenBank MW119276).** A scheme of the design and assembly appears in Supplementary Fig. S1. Details of PCR amplifications, templates, oligonucleotides and size/functions of each amplified unit are depicted in Supplementary Tables S2 and S3. The set of 9 structural PCRs and 5 linker PCRs used in this assembly were obtained as follows: five PCR fragments (~ 4 kb-long each) were produced to amplify the Tra1 and Tra2 regions of plasmid RP4. The two fragments representing Tra1 (PCR-1 and -2) were designed to have an overlapping region of 0.8 Kb. The three fragments from Tra2 (PCR-3, -4 and -5) were designed such that the PCR-4 overlapped with the other two fragments by 0.5 Kb and 0.8 Kb, respectively. Additional two PCR fragments were amplified from pBAMD1-2 to obtain a plasmid backbone devoid of the oriT: PCR-6 provided the R6K origin of replication, the ME-I edge of the Tn5 cassette and a part of the MCS (Multi Cloning Site) ending in the EcoR-I site; while the PCR-7 provided the *trpA* transposase gene, the ApR and KmR resistance genes, the ME-O edge of the Tn5 cassette and part of the MCS ending in the Pst-I site. The *xytS-P_m* promoter system was obtained from pSEVA258 (PCR-8). An additional fragment, consisting of genes necessary for plasmid replication (CEN6/ARS209) and selection (URA) within *S. cerevisiae*, was amplified from pSEVA222Sβ (PCR-9). Finally, 5 linker PCRs were produced to provide bridges of homology between the plasmid structural regions that would neighbor each other in the final design. Every linker was constructed via two PCR reactions of ~ 0.5 Kb each (hemi-linkers). A ~ 20 bp tail of homology was included in one of the primers, allowing the fusion of both hemi-linkers into a single molecule of ~ 1.0 Kb by sewing PCR. The 14 DNA fragments (PCR-1 to 9 and Linker-1 to 5) were transformed in *S. cerevisiae CRY1-2 and plasmid DNA from two selected clones was electroporated into *E. coli* TransforMax™ EC100D™ pir⁺ electrocompetent cells (Cat. Nº ECP09500- Lucigen, Middleton, USA) following manufacturer recommendations. This strain provides the pir gene in *trans*, allowing the propagation of plasmids with a R6K origin of replication. Selection was performed in LB-Ap Km agar plates, and one colony from each transformation was checked by miniprep and restriction analysis. Both clones were fully sequenced to ensure the accuracy of the construct. The resulting DNA diverged from the published sequences in 4 bases i.e. a T insertion in position 7611 (potential bi-directional terminator in Tra1 Core, downstream the gene *traM*), a T→C change in position 8906 (intergenic region in *xytS-P_m*).
a G→A change at site 15821 (Tra2 Core, Gly\textsubscript{67}Asp change in \textit{trbH}) and a G→A change at site 17095 (Tra2 Core, silent mutation in Gln\textsubscript{329} of \textit{trbI}). These changes had no apparent effect in the activity of the genetic device. It is worth mentioning that the design was such that all necessary recombination events had DNA substrates with homology arms of 0.5-0.8 Kb to facilitate the assembly procedure in \textit{S. cerevisiae} cells. This design included a sewing PCR between PCR-6 (ori R6K) and PCR9 (CEN6/ARS209-URA), allowed by the tail of homology (20 bp) included in the primer oriR6K-F. Therefore, the generated fragment PCR-6+9 could be connected with PCR-1 (Tra1-1) by Linker-1. However, this sewing PCR could not be obtained. The assembly of pTRANS with the separated fragments PCR-6 and PCR-9 was successful just relying on the short homology included in PCR-6 (denoted by a blue asterisk in Supplementary Fig. S1)
**Figure S1.** Detailed strategy of pTRANS construction.

The left panels show the plasmid templates used for amplifications. Inside each plasmid, straight and stroked bars represent, respectively, structural and hemi-linker PCRs. Numbers in circles refer to PCR/hemi-linker annotation found in Table S2. On the right, the assembly scheme of pTRANS via homologous recombination is shown inside a *S. cerevisiae* cell, with the structural PCRs in the inner circle and the linker PCRs in the external circle. All recombination events were provided with homology regions of 0.5-0.8 Kb, with the exception PCR6/PCR9 connection: the blue asterisk indicates the short homology region of 20 bp included in the primer oriR6K-F of PCR6. The pTRANS final construct is also shown in the left-bottom panel.
figure S2. Phenotypic characterization of donor and recipient strains used in this study.

Growing phenotypes of *P. putida*-TRANS#9 (donor, D), *P. putida* JS40 (receptor, R) and *P. putida* EM42 ancestral strain were assayed in different M9 solid media. Regardless the addition of uracil, donor strain grew both in citrate and glucose carbon sources, showing Km resistance and Gm sensitivity. Receptor strain was Km sensitive, had uracil-dependant growth and showed only residual growth on glucose carbon source, supporting Gm concentrations up to 50 µg/ml. Green fluorescence of *P. putida* JS40 can be seen in M9-Citrate-Ura under UV light.
**Figure S3.** PCR analysis of *pyrF* and *edd* genes in *P. putida* JS40 trans-conjugants.

**A.** Twenty *P. putida* JS40 trans-conjugants (lanes 1 to 20) isolated in M9-Citrate-Gm50 media (*pyrF* transfer- selected across several experiments with donors *P. putida*-TRANS#9 and #18) were PCR checked with primers *pyrF*-F/*pyrF*-R. All PCRs showed the expected amplicon size for the intact *pyrF* gene (1.2 Kb), confirming the transfer of the marker gene. Control amplifications of #9 donor strain (D) and *P. putida* JS40 receptor strain (R) showed, respectively, the expected sizes of 1.2 Kb (wild-type gene) and 0.5 Kb (*ΔpyrF* deletion mutant). M stands for Bio-Rad EZ Load 500 bp Molecular Ruler.

**B.** Twenty *P. putida* JS40 trans-conjugants (lanes 1 to 20) isolated in M9-Glucose-Ura-Gm50 media (*edd* transfer- selected across several experiments with donors *P. putida*-TRANS#9 and #18) were PCR checked with primers *edd*-check-F/*edd*-check-R. All PCRs showed the expected amplicon size for the intact *edd* gene (1.5 Kb), confirming the transfer of the marker gene. Control amplifications of #9 donor strain (D) and *P. putida* JS40 receptor strain (R) showed, respectively, the expected sizes of 1.5 Kb (wild-type gene) and 0.5 Kb (*Δedd* deletion mutant). M stands for Bio-Rad EZ Load 500 bp Molecular Ruler.
### Supplementary Table S1. Bacterial strains and plasmids used in this work.

| Strain or plasmid | Relevant characteristics | Reference or source |
|-------------------|--------------------------|---------------------|
| **Escherichia coli** |
| CC118             | Cloning host; Δ(ara-leu) araD ΔlacX74 galE, galK phoA thiE1 rpsE(SpR), rpoB(RifR), argE(Am), recA1 | 11 |
| CC118 λpir        | CC118, λpir lysogen      | 12 |
| DH5αλpir          | Cloning host for R6K-derived plasmids; supE44, ΔlacU169 (φ80 lacZΔM15), hsdR17 (rk-mk+), recA1, endA1, thi1, gyrA, relA, lysogenic λpir | Lab collection |
| HB101             | Helper strain used for conjugation; F-, λ-, hsdS20 (rB- mB-), recA13 leuB6(Am), araC14 ∆(gpt-proA)62, lacY1 galK2(Oc), xyl-5, mtl-1, thi1, rpsL20 (SmR), glnX44 (AS) | 13 |
| TransforMax™ EC100D™ pir* | F- mcrA ∆(mrr-hsdRMS-mcrBC) (φ80 lacZΔM15) ΔlacX74 recA1 endA1 araD139 ∆(ara,leu)7697 galU galK λ− rpsL NupG pir+(DHFR). | Lucigen® |
| **Pseudomonas putida** |
| EM42              | KT2440 derivative; Δprophage1 Δprophage4 Δprophage3 Δprophage2 ΔTn7 ΔendA-1 ΔendA-2 ΔhsdRMS Δflagellum ΔTn4652 | 4 |
| TA280             | EM42 derivative; rpsL (SmR), rpoB (RifR), gyrA (NaF) | This work |
| TRANS#X           | TA280 derivatives harboring mini-Tn5-TRANS insertions, KmR, rpsL (SmR), rpoB (RifR), gyrA (NaF) | This work |
| EM42 ΔpyrFΔedd    | EM42 derivative; ΔpyrF, Δedd | 6 |
| JS40              | EM42 derivative; ΔpyrF, Δedd, mini-Tn7 transposon harboring P<sub>EM7-gfp</sub>, GmR | This work |
| **Saccharomyces cerevisiae** |
| CRY1-2            | MATα, ura3, Δcyh2R | Lab collection |
| Plasmids                  | Description                                                                                                                                                                                                 | Source          |
|--------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|
| pRK600                   | Helper plasmid used for conjugation; oriV(ColE1), RK2 (mob+ tra+); Cm<sup>R</sup>                                                                                                                       | Lab Collection |
| RP4                      | Wild type conjugative plasmid; Tra1-Tra2 Cores; oriV(RK2); Tc<sup>R</sup>; Km<sup>R</sup>; Ap<sup>R</sup>                                                                                         | This work       |
| pSEVA222S<sub>P</sub>    | Yeast shuttle vector; oriV(RK2); cargo [lacZ<sub>α</sub>-pUC19/I-SceI]; gadget β; Km<sup>R</sup>                                                                                                     | This work       |
| pTRANS                   | Mini-Tn5-TRANS delivery plasmid; oriV(R6K); Ap<sup>R</sup>; Km<sup>R</sup>; CEN6-ARS209-URA3; TRANS module (Tra1-Tra2 Cores); xy/S-P<sub>m</sub> expression system | This work       |
| pRS416                   | Yeast shuttle vector; oriV(ColE1); lacZ<sub>α</sub>; CEN6-ARS209; URA3; Ap<sup>R</sup>                                                                                                                   | This work       |
| pSEVA221                 | Cloning vector; oriV(RK2); standard multiple cloning site; Km<sup>R</sup>                                                                                                                                | This work       |
| pSEVA212S                | Cloning vector; oriV(RK2); cargo [lacZ<sub>α</sub>-pUC19/I-SceI]; Km<sup>R</sup>                                                                                                                      | This work       |
| pBAMD1-2                 | Mini-Tn5 delivery plasmid; oriV(R6K); Ap<sup>R</sup>; Km<sup>R</sup>                                                                                                                                       | This work       |
| pSEVA238                 | Expression vector; oriV(pBBR1); cargo [xyt/S-P<sub>m</sub>]; standard multiple cloning site; Km<sup>R</sup>                                                                                              | This work       |
| pTn7-M-PEM7-GFP          | pTn7-M derivative with P<sub>Emr-gfp</sub> in the mini-Tn7 transposon; oriV(R6K); Km<sup>R</sup> Gm<sup>R</sup>                                                                                               | This work       |
| pTNS2                    | Helper plasmid for mini-Tn7 transposition; oriV(R6K), TnsABC+D specific transposition pathway; Ap<sup>R</sup>                                                                                          | This work       |
### Supplementary Table S2. Primers, templates and PCR conditions of pSEVA222S<sub>β</sub> and pTRANS assemblies.

#### pSEVA222S<sub>β</sub> Assembly

| PCR name  | Primers                          | Template         | Tm/Te     | Size (bp) | Fragment amplified                |
|-----------|----------------------------------|------------------|-----------|-----------|-----------------------------------|
| PCR-A     | lacZa-AscI-F lacZa-AscI-R        | pSEVA212S        | 66 °C/ 30'' | 781       | lacZa-pUC19/I-SceI               |
| PCR-B     | CEN6-F/ CEN6-R                   | pRS416           | 57 °C/ 30'' | 510       | CEN6/ARS209                       |
| PCR-C     | URA3-F/URA3-intR                 | pRS416           | 55 °C/ 30'' | 219       | URA3-partial                      |
| PCR-D     | URA3-intF/ URA3-R                | pRS416           | 55 °C/ 30'' | 924       | URA3-partial                      |
| PCR-E     | URA3-F/ URA3-R                   | PCR-C+PCR-D      | 55 °C/ 30'' | 1101      | URA3 (cured of Pest-I restriction site) |
| PCR-F     | RK2-Km-F/RK2-Km-R                | pSEVA2214        | 60 °C/ 2'  | 3440      | ori RK2 and Km<sup>β</sup> cassette |

#### pTRANS Assembly

| PCR name  | Primers                          | Template         | Tm/Te     | Size (bp) | Fragment amplified                |
|-----------|----------------------------------|------------------|-----------|-----------|-----------------------------------|
| PCR-1     | Tra1-F1 / Tra1-R1                | RP4              | 60 °C/ 2'  | 3995      | Tra1-1                            |
| PCR-2     | Tra1-F2 / Tra1-R2                | RP4              | 60 °C/ 2'  | 3852      | Tra1-2                            |
| PCR-3     | Tra2-F1 / Tra2-R1                | RP4              | 60 °C/ 2'  | 3766      | Tra2-1                            |
| PCR-4     | Tra2-F2 / Tra2-R2                | RP4              | 60 °C/ 2'  | 4106      | Tra2-2                            |
| PCR-5     | Tra2-F3 / Tra2-R3                | RP4              | 61 °C/ 2'  | 4064      | Tra2-3                            |
| PCR-6     | oriR6K-F/ oriR6K-R               | pBAMD1-2         | 70 °C/ 30'' | 586       | ori R6K and ME-I                 |
| PCR7      | pBAMD-F/ pBAMD-R                 | pBAMD1-2         | 60 °C/ 2'  | 3799      | TrpA, Km<sup>β</sup> and Ap<sup>μ</sup> and ME-O |
| PCR-8     | xylS-F / Pm-R                    | pSEVA258         | 60 °C/ 1'  | 1987      | xylS-Pm                           |
| PCR-9     | CEN6-F / URA3-R                  | pSEVA222S<sub>β</sub> | 50 °C/ 1' | 1611      | CEN6/ARS209 and URA3              |
| Hemi-Linker-1.1 (=PCR6) | oriR6K-F/ oriR6K-R          | pBAMD1-2         | 70 °C/ 30'' | 586       | First half of Linker-1            |
| Hemi-Linker-1.2 | L1-F2 / L1-R2               | RP4              | 60 °C/ 30'' | 580       | Second half of Linker-1           |
| Hemi-Linker-2.1 | L2-F1 / Tra1-R2          | RP4              | 60 °C/ 30'' | 511       | First half of Linker-2            |
| Hemi-Linker-2.2 | L2-F2 / L2-R2            | pSEVA258         | 60 °C/ 30'' | 669       | Second half of Linker2            |
| Hemi-Linker-3.1 | L3-F1 / Pm-R              | pSEVA258         | 60 °C/ 30'' | 581       | First half of Linker-3            |
| Hemi-Linker-3.2 | L3-F2 / L3-R2          | RP4              | 60 °C/ 30'' | 546       | Second half of Linker3            |
| Hemi-Linker-4.1 | L4-F1 / Tra2-R3         | RP4              | 59 °C/ 30'' | 631       | First half of Linker-4            |
| Hemi-Linker-4.2 | L4-F2 / L4-R2         | pBAMD1-2         | 60 °C/ 30'' | 584       | Second half of Linker-4           |
| Hemi-Linker-5.1 | L5-F1 / pBAMD-R       | pBAMD1-2         | 60 °C/ 30'' | 494       | First half of Linker-5            |
| Hemi-Linker-5.2 | L5-F2 / L5-R2       | pSEVA222S<sub>β</sub> | 60 °C/ 30'' | 674       | Second half of Linker-5           |
| Linker-1  | oriR6K-F / L1-R2              | Linkers 1.1+1.2  | 60°C / 45'' | 1169      | Sewing PCR for Linker-1           |
| Linker-2  | L2-F1 / L2-R2                 | Linkers 2.1+2.2  | 60°C / 45'' | 1160      | Sewing PCR for Linker-2           |
| Linker-3  | L3-F1 / L3-R2                 | Linkers 3.1+3.2  | 60°C / 45'' | 1079      | Sewing PCR for Linker-3           |
| Linker-4  | L4-F1 / L4-R2                 | Linkers 4.1+4.2  | 60°C / 45'' | 1192      | Sewing PCR for Linker-4           |
| Linker-5  | L5-F1 / L5-R2                 | Linkers 5.1+5.2  | 60°C / 45'' | 1147      | Sewing PCR for Linker-5           |
### Supplementary Table S3. Oligonucleotides used in this study.

| Name             | Sequence (5’ → 3’)
|------------------|-------------------|
| lacZa-AscI-F     | ggtctcaagaagatcctgtatatcttacGCGC GCCCCAGCTGCTCTAG |
| lacZa-AscI-R     | attttaatattatattttataatgctagctaaaggG GGACCCTGAGTTCTCACC |
| CEN6-F           | cttttcatcgcctataaaaatattataatttaattattttatatagcacgtgatgaaaagG GGGACCCCTGAGTTCTCACC |
| CEN6-R           | GATCGCTTGCTGTAAC |
| URA3-F           | taaaaagatacagagaggcgtgaagttacagggcaaggG tacCCCTTTCAATTCATCATTTTTTTTATT |
| URA3-intR        | GATTATCTCTCGTTCCTGCAATTTTT TTCTCTGAGTTCTCAG |
| URA3-intF        | CCAACTGACAGAAACAAAATTGCAGAAGAAGATAAAT |
| URA3-R           | tggatatgaactcagatcggcgctgattcagggcagG tacCCCTTTCAATTCATCATTTTTTTTATT |
| RK2-Km-F         | ATTTAAATTTGCTCTCAAATCTCTGATG |
| RK2-Km-R         | GGCUGCGCGGTAGAAAAAGATCAAAGG ATCTTTC |
| Tra1-F1          | ATATCCCCCTACCCTCACC |
| Tra1-R1          | GAAAACGGCTCTATCTCGAG |
| Tra1-F2          | GATCCGCTCCTTTGAACCTCTG |
| Tra1-R2          | TCAAAACAGCCAGCTAAGG |
| Tra2-F1          | TTGCCCTGCGATAATGTCG |
| Tra2-R1          | CCGAGGTTCATCGAGCTACTG |
| Tra2-F2          | TCGGCCACACCTTTATGTC |
| Tra2-R2          | GAGTTGATGCCGGATAAGC |

*Usage/Source:*
- To amplify the lacZα-pUC19/I-SceI cargo from pSEVA212S; homology tail with RK2-Km-R
- To amplify the lacZα-pUC19/I-SceI cargo from pSEVA212S; homology tail with CEN6-F
- To amplify the CEN6/ARS209 cassette from pRS416, removing a Swa-I site; homology tail with lacZα-AscI-R
- To amplify the CEN6/ARS209 cassette from pRS416
- To amplify the URA3 cassette from pRS416; homology tail with CEN6-R
- To amplify the URA3 cassette from pRS416, removing a Pst-I restriction site
- To amplify the URA3 cassette from pRS416; homology tail with RK2-Km-F
- To amplify the RK2 origin of replication and KmR cassette from pSEVA2214
- To amplify the RK2 origin of replication and KmR cassette from pSEVA2214
- To amplify the Tra1-1 region from RP4
- To amplify the Tra1-1 region from RP4
- To amplify the Tra1-2 region from RP4
- To amplify the Tra1-2 region from RP4 and Linker-2
- To amplify the Tra2-1 region from RP4
- To amplify the Tra2-1 region from RP4
- To amplify the Tra2-2 region from RP4
- To amplify the Tra2-2 region from RP4
|            | Sequence                                                                 | Function                                                                 |
|------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Tra2-F3    | CATGGGTTCCGAAGGAGTAAG                                                     | To amplify the Tra2-3 region from RP4                                      |
| Tra2-R3    | CGCTTTTTTCAAGTTGCATGGTC                                                   | To amplify the Tra2-3 region from RP4 and Linker-4                         |
| oriR6K-F   | ttatagcacgtgataaagGGCCGGCGGATC TGAAGATCAGCAGTTCAAC                       | To amplify the ori R6K and ME-1 region from pBAMD1-2, homology tail with CEN6-F. Also for Linker-3 |
| oriR6K-R   | GAATCCGCGCGGCGGCGG                                                     | To amplify the ori R6K and ME-1 region from pBAMD1-2. Also for Linker-3    |
| pBAMD-F    | CTGCAGGCATGCAAGCTTG                                                      | To amplify trpA gene, Km<sup>R</sup> and Ap<sup>R</sup> cassettes and ME-O region from pBAMD1-2 |
| pBAMD-R    | GAAAAGGACAAAACCGCTGTCC                                                 | To amplify trpA gene, Km<sup>R</sup> and Ap<sup>R</sup> cassettes and ME-O region from pBAMD1-2. Also for Linker-5 |
| xylS-F     | ACGTTCTGTAATCAAGCCACTTC                                                 | To amplify the xylS-P<sub>m</sub> cassette from pSEVA258                  |
| Pm-R       | CATGGTCATGACTCCATTATTATTGTTC                                             | To amplify the xylS-P<sub>m</sub> cassette from pSEVA258 and Linker-3     |
| L1-F2      | ccgcggccgcgaattcATATCCCCCTACC CTCACC                                    | To amplify the Linker-1 to connect the ori R6K region with Tra1-1 region, homology tail with oriRK6-R |
| L1-R2      | GAGGGGATCACGATATGAGC                                                 | To amplify the Linker-1 to connect the ori R6K region with Tra1-1 region   |
| L2-F1      | TGCTATGACGCCAGACATTG                                                  | To amplify the Linker-2 to connect Tra1-2 region with the xylS-P<sub>m</sub> cassette |
| L2-F2      | cgttaagctgcctgaattcaGCCTCGTAATCAA GCCACTTC                               | To amplify the Linker-2 to connect Tra1-2 region with the xylS-P<sub>m</sub> cassette, homology tail with Tra1-R2 |
| L2-R2      | CAAGGCCGACCTGACCTATTC                                                  | To amplify the Linker-2 to connect Tra1-2 region with the xylS-P<sub>m</sub> cassette |
| L3-F1      | TCCATGAGCAAACGTGAACAG                                               | To amplify the Linker-3 to connect the xylS-P<sub>m</sub> cassette with Tra2-1 |
| L3-F2      | gaaacaataataaactgtcatgcaccattTTGCC GTGCAGTAATGTTC                         | To amplify the Linker-3 to connect the xylS-P<sub>m</sub> cassette with Tra2-1, homology tail with Pm-R |
| L3-R2      | GCCAATGACGAGGATGTTC                                                  | To amplify the Linker-3 to connect the xylS-P<sub>m</sub> cassette with Tra2-1 |
| L4-F1      | AGGCCAGCGATAACGTCTC                                                  | To amplify the Linker-4 to connect Tra2-3 with the TrpA, Km<sup>R</sup> and Ap<sup>R</sup> region of pBAMD1-2 |
|   | Sequence                          | Function                                                                 |
|---|-----------------------------------|-------------------------------------------------------------------------|
| L4-F2 | gaccatgcaactgaaaaagcgtgacgca TTGAAGCTTTT   | To amplify the Linker-4 to connect Tra2-3 with the TrpA, Km<sup>R</sup> and Ap<sup>R</sup> region of pBAMD1-2, homology tail with Tra2-R3 |
| L4-R2 | TATGCAGACGCAGCAGAAAC              | To amplify the Linker-4 to connect Tra2-3 with the TrpA, Km<sup>R</sup> and Ap<sup>R</sup> region of pBAMD1-2 |
| L5-F1 | GAAAGAACTGACGCAGCTTTC             | To amplify the Linker-5 to connect TrpA, Km<sup>R</sup> and Ap<sup>R</sup> region of pBAMD1-2 with the CEN6/ARS209 cassette  |
| L5-F2 | gacacgcttttctctttcGGGTAATAACTGA TATATATAATTGACGCTTC | To amplify the Linker-5 to connect TrpA, Km<sup>R</sup> and Ap<sup>R</sup> region of pBAMD1-2 with the CEN6/ARS209 cassette, homology tail with pBAMD-R  |
| L5-R2 | TAAAGGCATTATCCGCCAAG              | To amplify the Linker-5 to connect TrpA, Km<sup>R</sup> and Ap<sup>R</sup> region of pBAMD1-2 with the CEN6/ARS209 cassette  |
| MEO-Km-extF | CTCTGTTTTTCAGAAATATGCAT         | Used in arbitrary PCR round 1 for Tn-5 localization 2  |
| ARB6 | GGCACCGCTGCACTAGTACNNNNNNNNNNNACGCC | Used in arbitrary PCR round 1 for Tn-5 localization 2  |
| MEO-Km-intF | ATCTGATGCTGGATGAATTTTC         | Used in arbitrary PCR round 2 for Tn-5 localization 2  |
| ARB2 | GGCACGCGTGCACTAGTAC              | Used in arbitrary PCR round 2 for Tn-5 localization 2  |
| SR | G*T*C*A*GACGCACAGGCATACTTTTA CCGAGGGACGATTCATGGTTCCTGCGG CTGGTTGTGTTAACAACAGGCGTGAC ACGCCACGACGCTTC | Recombineering oligo for rpsL gene: AAA (K43) changed to ACA (T43), mismatch A:G, confers Sm resistance 18  |
| rpsL-Fw | GACATGAAATGTTGCCGATG            | To amplify and sequence part of rpsL gene of P. putida 19  |
| rpsL-Rv | CTGGTTTTCCGTGGTTGAC            | To amplify and sequence part of rpsL gene of P. putida 19  |
| RR | TCCGAGAGGGTTTCTGGTCCAT GACAGGGAGAGCTGGTGGAAACG AAGAACTCT | Recombineering oligo for rpoB gene: CAG (Q518) changed to CTG (L518), mismatch A:A, confers Rif resistance 5  |
| rpoB-F | CCTGGTTACGCGCGGTACGGTG           | To amplify and sequence part of rpoB gene of P. putida 5  |
| rpoB-R | CGCCTCCTCCTACACGCGGTACG          | To amplify and sequence part of rpoB gene of P. putida 5  |
| NR | AACGAGAACGGCTGGGCCCATACGGA CGATGGATTGAGACCGCGATGGTGCC GGTCGGGTTGGA | Recombineering oligo for gyrA gene: GAC (D87) changed to AAT (N87), mismatches G:T  |
| Primer  | Sequence                        | Function                                                                 |
|---------|---------------------------------|--------------------------------------------------------------------------|
| gyrA-F  | GGCCAAAGAAATCCTCCCGTCAA         | To amplify and sequence part of gyrA gene of *P. putida*                 |
| gyrA-R  | AGCAGGTTGGGAATACGCGTGA          | To amplify and sequence part of gyrA gene of *P. putida*                 |
| pyrF-F  | CGAGGGCTATGATGAGTATC            | To amplify and sequence the pyrF gene (PP_1815) of *P. putida*           |
| pyrF-R  | GTCAGGTGAAGAGCAAAGAG            | To amplify and sequence the pyrF gene (PP_1815) of *P. putida*           |
| edd-check-F | TAAACCGCTCTACAATTAG          | To amplify and sequence the edd gene (PP_1010) of *P. putida* EM42       |
| edd-check-R | ACCAACGCAACCTTGTAG            | To amplify and sequence the edd gene (PP_1010) of *P. putida* EM42       |

(a) Lower case denotes homology tails with other oligos. Ns in primer ARB6 are degenerated positions (N=A,G,CT). Mutated Pst-I site (CTGCAA/TTGCAG) appears in red in primers URA3-intR/ URA3-intF and the mutated position is underlined. In CEN6-F, mutated Swa-I site (atttaaaa) appears in blue and the mutated position is underlined. Asterisks denote phosphorothioate bonds. Single changes introduced by recombineering oligonucleotides SR, NR and RR are highlighted in bold.

(b) A reference source only appears for primers designed in other works.

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