A New and Improved Host-Independent Plasmid System for RK2-Based Conjugal Transfer

Trine Aakvik Strand¹, Rahmi Lale¹*, Kristin Fløgstad Degnes², Malin Lando¹, Svein Valla¹

¹ Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway, ² Department of Biotechnology, SINTEF Materials and Chemistry, Trondheim, Norway

Abstract

Bacterial conjugation is a process that is mediated either by a direct cell-to-cell junction or by formation of a bridge between the cells. It is often used to transfer DNA constructs designed in *Escherichia coli* to recipient bacteria, yeast, plants and mammalian cells. Plasmids bearing the RK2/RP4 origin of transfer (oriT) are mostly mobilized using the *E. coli* S17-1/SM10 donor strains, in which transfer helper functions are provided from a chromosomally integrated RP4::Mu. We have observed that large plasmids were occasionally modified after conjugal transfer when using *E. coli* S17-1 as a donor. All modified plasmids had increased in size, which most probably was a result of co-transfer of DNA from the chromosomally located oriT. It has earlier also been demonstrated that the bacteriophage Mu is silently transferred to recipient cells by these donor strains, and both occurrences are very likely to lead to mutations within the recipient DNA. Here we report the construction of a new biological system addressing both the above mentioned problems in which the transfer helper functions are provided by a plasmid lacking a functional oriT. This system is compatible with all other replicons commonly used in conjugation experiments and further enables the use of diverse bacterial strains as donors. Plasmids containing large inserts were successfully conjugated and the plasmid modifications observed when *E. coli* S17-1 was used as donor were eliminated by the use of the new host-independent vector system.

Introduction

Due to its well established genetics and good transformation competence *Escherichia coli* is the most frequently used host for manipulation of DNA via a variety of recombinant DNA technologies. After the modifications have been made it might be necessary to transfer the constructs designed in *E. coli* to alternative hosts at high frequencies. This becomes relevant for example during construction of large numbers of transposon insertion mutants or for transfer of metagenomic libraries in functional screening studies across species barriers. Transformation of naked DNA is often inefficient, or sometimes even impossible, depending on the host of interest. The use of conjugation often solves these problems as the transfer system is mainly acting in a recipient-independent manner [1]. While the recipient-independency is an attractive feature, there also exist limitations due to the requirements of complex machinery and also due to protection systems in recipient cells, such as CRISPR and restriction-modification [2,3]. The *E. coli* strain S17-1 and its analogue SM10 are heavily used as donor strains in such transfer procedures, which is reflected by a very high citation frequency (nearly 5000 as of October 2013) of the paper in which these strains are described [4].

*E. coli* S17-1/SM10 contain a chromosomally integrated RP4 plasmid, which is essentially the same as the more studied broad-host-range self-transmissible IncP plasmid RK2 [3]. Conjugal transfer of plasmids based on this system requires the presence of an origin of transfer (oriT) in the plasmid to be transferred, as well as the gene products of two separate tra-clusters [6] which are provided in trans from the RP4 integrated in *E. coli* S17-1. A number of small and specialized oriT-containing vectors have been developed from the large RK2 plasmid (60 kb) [7], but other types of plasmids containing oriT may also be conjugated by *E. coli* S17-1/SM10 [8,9].

In spite of their extensive use there are several problems associated with the *E. coli* strains S17-1 and SM10: They both contain an active bacteriophage Mu genome (within the tetracycline resistance gene of RP4) which has been shown to mobilize itself into recipient strains [10,11]. This may cause problems as Mu DNA may randomly mutate the recipient genome and/or the transferred plasmid. Another demonstrated problem is that these strains not only mobilize oriT-carrying plasmids, but also their own chromosomal DNA to recipient strains at frequencies of 10⁻⁴ per donor cell [12]. Furthermore, there are also reports describing generation of plasmid modifications of unknown nature in conjugation experiments involving *E. coli* strains S17-1/SM10 [13,14].

In addition to these findings we here report that plasmids transferred from *E. coli* S17-1 to other bacterial species quite often become modified by insertion of DNA from the donor host chromosome, presumably as a result of mobilization of DNA from the active oriT within the inserted RP4. This represents a rather serious problem as it very likely can lead to inactivation of genes in
such transferred plasmids. There have been established alternative conjugation systems which address some of the above mentioned problems separately, such as a modified *E. coli* S17-1 strain in which the Mu genome has been inactivated [11]. In this study we present a new and improved system for conjugal transfer of mobilizable plasmids which overcomes both the problems of bacteriophage Mu and chromosomal DNA mobilization from the donor. This system is constructed in a way that all the functions required for conjugal transfer are present on a broad-host-range (RK2-compatible) plasmid, a feature that allows the use of diverse bacterial hosts as donors for conjugation of oriT-containing constructs.

**Materials and Methods**

**Bacterial strains, plasmids and growth media**

The bacterial strains and plasmids used in this study are described in Table 1. The fosmid vectors used in the conjugation experiments are from a previous study and were constructed using the broad-host-range cloning vector pRS44 [15]. This vector harbours two replications: ori2, leading to single plasmid copy in *E. coli*; and oriV, leading to high copy-number in strain EPI300 when induced. This strain harbours a gene encoding a high copy-number replication protein for oriV; TrfA, which is under a tightly regulated inducible promoter on its chromosome. Upon induction the vector copy-number increases from a single-copy to high copy-number as replication then occurs via oriV [16].

The growth media used were Lysogeny Broth (LB, 5 g yeast extract, 5 g NaCl and 10 g tryptone per litre) and Lysogeny Agar (LA, LB supplemented with 20 g agar per litre) for *E. coli* strains, LB and Difco Pseudomonas Isolation agar (PIA) for *Pseudomonas fluorescens*, and Yeasy Mold (YM) broth and YM agar for *Xanthomonas campestris*. Antibiotics were used at the following concentrations when relevant: ampicillin, 100 µg mL⁻¹ (*E. coli*); chloramphenicol, 12.5 µg mL⁻¹ (*E. coli*), 30 µg mL⁻¹ (*X. campestris*); kanamycin, 50 µg mL⁻¹ (*E. coli* and *P. fluorescens*); tetracycline 10 µg mL⁻¹ (*E. coli*), 15 µg mL⁻¹ (*X. campestris*) or 25 µg mL⁻¹ (*P. fluorescens*). Expression of βifA (wt, i.e. low copy-number) from *PmG5* in the recipient *P. fluorescens* and *X. campestris* strains was induced by addition of m-toluate at 0.5 mM, enabling replication of the transferred fosmids from oriV. Counter selection of sacB containing strains was done using sucrose at 5% (w/v). Clones in *E. coli* EPI300 were switched from single-copy to high copy-number by L-arabinose induction at 0.01% (w/v).

**Standard DNA manipulations and conjugative matings**

Routine DNA manipulations and agarose gel electrophoresis were performed according to the methods of Sambrook and Russel [17], or by using commercially available kits. DNA sequencing was performed using the Big Dye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems). Transformations of *E.coli DH5α*, ER2566 and EPI300 were performed according to the RbCl transformation protocol (New England Biolabs) or through electroporation according to Sambrook and Russel [17] (13 V cm⁻¹, 200 µΩ, 25 µF).

Conjugative matings were performed as follows: cells from 2 mL exponential phase growing cultures (OD = 0.4) of donor- and recipient strains were mixed, concentrated after centrifugation and deposited onto LA without antibiotic selection (30°C, overnight). The mixtures were then plated on appropriate selective media and incubated at 30°C for 48 h, for *P. fluorescens* or 72 h for *X. campestris*.

Plasmids were isolated from cultures of *P. fluorescens* and *X. campestris* using commercial plasmid isolation kits and isopropanol precipitation, and retransformed into *E. coli* EPI300 by electroporation.

**Vector constructions**

The suicide vector pTA10 was constructed through ligation of the narrow host range replicon oriR6K, the sacB gene and a chloramphenicol resistance gene, using the *pir*-expressing *E. coli* strain S17-1pir as host. The deletions within RK2 resulting in plasmids pTA17 and pTA19 were performed through homologous recombination, using two pTA10-derivatives (pTA16 and pTA15, respectively) carrying PCR-fragments homologous to the regions flanking the deleted segments (next paragraph). The PCR-fragments within pTA15 were amplified with the following primers (restriction sites are underlined): PCR-fragment oriT1: OriT1fwdXhol: 5'-TTTCTCCATGGCGATACGGGT-CATGGATGGG-3' and OriT1revEcoRI 5'-TTTGAATTCCGGCAACGCGATGCGTGATGA-3'; PCR-fragment oriT2: OriT2fwdEcoRI: 5'-TTTGAATTCTCGAGCGGTGGATACGACC-3' and OriT2revNheI 5'-TTTGGCATGCGTGCAGATGTGCAGATGTA-3'. Counter selection of chloramphenicol-resistant transformants. As pTA16 were amplified from RK2 using the following primers: PCR fragment Km-1: Km1fwdXhol: 5'-TTTCTCCAGACACGACGCACACAGGTATGATG-3' and Km1revEcoRI 5'-TTTGAATTCTGAGGGTGATACGACC-3'; PCR-fragment Km-2: Km2fwdEcoRI: 5'-TTTGAATTCTGCCGTTGGAAC1-GTC-3' and Km2revNheI 5'-TTTGGCATGCGTGCAGATGTGCAGATGTA-3'. After amplification, the oriT-1 fragment was ligated to the oriT-2 fragment at the EcoRI sites, after which the oriT-1/oriT-2 fragment was ligated into the Xhol-NheI sites of pTA10. The procedure was repeated for the Km-1 and Km-2 PCR fragments.

**Homologous recombination for targeted deletions within RK2**

For inactivation of oriT, *E. coli* ER2566 cells (recA) containing plasmid RK2 were first transformed with pTA16, followed by selection of chloramphenicol-resistant transformants. As pTA16 cannot replicate extra-chromosomally in this strain, these represented cells in which recombination had happened between either the oriT-1 or the oriT-2 fragment. The cells were first cultivated in the presence of chloramphenicol overnight, then, after re-inoculation (0.5% overnight-culture to fresh media), for at least 6 hours in the absence of selection. Different dilutions were next plated on LA plates containing sucrose, selecting for cells not containing sacB, i.e. where also the second cross-over had occurred (at the remaining oriT fragment). Correct alteration was confirmed through PCR reactions and sequencing, and in addition the new RK2 derivative (pTA17) was confirmed being conjugation deficient (due to inactivation of oriT).

Construction of the kanamycin sensitive derivative of pTA17 (pTA19) was performed similarly as described for pTA17, but with the use of pTA15 instead of pTA16.

**Construction of pTA-Mob**

The mobilization plasmid pTA-Mob was constructed from pTA19 by first removing the Asel-AvrII fragment (9.4 kb) containing the replication origin, oriV, and the ampicillin and tetracycline resistance genes. An Asel-AvrII fragment containing the broad-host-range replicon pBBR1, as well as a gentamycin resistance gene was next ligated into the same restriction sites. This pBBR1rep-Gm' fragment was amplified from plasmid pBBR1MCS-5 [18] in two PCR reactions using the following primer pairs (restriction sites are underlined): Gent-Fwd: 5'
Table 1. Bacterial strains and plasmids used in this study.

| Bacterial strain or plasmid | Properties* | Source or Reference |
|-----------------------------|-------------|---------------------|
| E. coli DH108               | F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δara, leu7697 galU galK- rpsL nupG | Invitrogen |
| EPI300 Phage T1-resistant and lacZ' strain with L-arabinose induced chromosomally expressed TrfA ( F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δara, leu7697 galU galK- rpsL nupG trfA tonA dhfrI ) | | [16] |
| ER2566 F- λ Tn7::miniTn10-TetS2 Rgb4-210::Tn10 (TetS) endA1 [dcm] | | NEB |
| S17-1 pro+, res+ (hsdR17 (ik- mK+)) recA+ with an integrated RP4-2-Tc::Mu-Km::Tn7, Tp' | | [4] |
| S17-1(lpir) λpir lysogen of strain S17-1 | | [21] |

**Pseudomonas fluorescens**

| NCIMB 10525 | Pseudomonas fluorescens wild type | NCIMB |
| NCIMB10525::TnRS48 | Derivative of NCIMB 10525 with transposon TnRS48 integrated into the chromosome | [15] |

**Xanthomonas campestris**

| B100-152 | Spontaneous xanA exopolysaccharide-negative mutant | [22] |
| B100-152::TnRS48 | Derivative of B100-152 with transposon TnRS48 from pRS48 integrated into the chromosome | [15] |

**Plasmids**

| 37, 67, 83 | Three different pRS44 fosmid clones carrying 35 kb inserts, Cm', Km' | This work |
| pBBR1MCS-5 | Cloning vector containing the broad-host-range replicon pBBR1, 4.8 kb, Gm' | [18] |
| pLITMUS28 | General cloning vector, 2.8 kb, Ap' | NEB |
| pRS44 | Broad-host-range combined fosmid and BAC cloning vector, 10.3 kb, Cm', Km' | [15] |
| pRS48 | Suicide vector with a mini-Tn5 transposon for insertion of the traA gene under PmG5/lys control, Ap', Tc', 10.5 kb | [15] |
| pTA10 | Suicide vector containing the oriR6K replicon and sac8, Cm', 3.8 kb | This work |
| pTA15 | Derivative of pTA10 containing two PCR fragments Km-1 and Km-2 (see text), Cm', 5.4 kb | This work |
| pTA16 | Derivative of pTA10 containing two PCR fragments oriT-1 and oriT-2 (see text), Cm', 5.4 kb | This work |
| pTA17 | Derivative of RK2, oriT', Ap', Km', Tc', 60.0 kb | This work |
| pTA19 | Derivative of pTA17, oriT', Km', Ap', Tc', 59.5 kb | This work |
| pTAA4/pTA-Mob | pTA19 derivative without the 9.4 kb AseI-AvrII fragment, containing instead a 2.8 kb pBBR1-Gm' fragment, Gm', 57.2 kb | This work |
| RK2 | Ap', Km', Tc', 60.1 kb | [6] |

*Ap*: ampicillin resistance; Cm': chloramphenicol resistance; Gm': gentamycin resistance; Km': kanamycin resistance; Tc': tetracycline resistance.

5'-CGTATTTGCAATTATCCACCTGGCCGCGGCGTGTGAC-3' and Gent-Rev: 5'-GCAGTTTCTGGCGACATGGAAGCCATC-3', pBBR1-Fwd: 5'-CGAATTCATACCCACCGGCTC-3' and pBBR1-Rev: 5'-TCCTAGGTTAAAGCGCTGCTAGC-3'.

Results and Discussions

Detection of plasmid-modifications after conjugal transfer from E. coli strain S17-1 to Pseudomonas fluorescens and Xanthomonas campestris

In an extension of the previously reported inter-species transfer experiments with fosmids from a metagenomic library [15], we discovered that some of the fosmids had increased in size after being conjugally transferred to P. fluorescens and X. campestris (see Materials and Methods for information about the fosmids). For analyses of the plasmids within transconjugants of P. fluorescens and X. campestris, the plasmids needed first to be retransformed into E. coli EPI300 in order to obtain high quality plasmid DNA. This step also ensures that the analysis is performed on only one fosmid. Fosmid preparations of the transferred/retransformed fosmids were then digested with restriction endonuclease HindIII and the resulting fragments were separated by agarose gel electrophoresis. The digestion patterns were then compared to the corresponding results obtained from the original plasmid preparations from the E. coli donor strains (Figure 1). Lanes 1-3 show a case where the restriction fragment band patterns for a randomly selected plasmid, designated 62, remain the same after conjugal transfer both to P. fluorescens and X. campestris. However, for another plasmid, designated 83, the restriction fragment band patterns were altered after being conjugated to P. fluorescens (lanes 5-7) (original fosmid band pattern in lane 4). Of the plasmids obtained after conjugation of fosmid 83 to X. campestris, one appeared very similar to the original fosmid in the E. coli donor (lane 8), while the band pattern originating from another X. campestris transconjugant was clearly different (lane 9). A final example is illustrated by...
transfer of a fosmid designated 37 (lane 10 shows the original fosmid). No obvious differences could be observed for the fosmid after transfer to *P. fluorescens* (lane 11), but the fosmids in lanes 12 (from *P. fluorescens*) and 13 (from *X. campestris*) were clearly different from the one in the donor. Notably, these two lanes display indistinguishable restriction band patterns even though the plasmids had been transferred to two different species, indicating that the modifications were originating from the donor.

The experiments described above revealed that fosmid modifications occasionally occur for a given fosmid, that each individual transfer event can lead to a different outcome for a given fosmid, that passage through two different hosts can apparently lead to the same modification, and finally that the modifications always involve increase in the fosmid size.

**Analysis of the inserted DNA**

To characterize the nature of the apparently inserted DNA, ten such altered DNA bands were excised from the agarose gel, purified, ligated into pLiti28 and end-sequenced. Three of the obtained ten sequences did not give any significant hits against the public databases when BLAST analysis was performed, indicating that these were originating from the metagenomic insert DNA. Analyses of the remaining seven sequences revealed that four of these originated from the *E. coli* donor strain chromosome, all within a region of ca. 35 kb, whereas the last three fragments were from a gene within the Tn7 transposon. Historically the Tn7 was used to inactivate the kanamycin resistance gene within RP4 during construction of the conjugation *E. coli* donor strain S17-1 [4].

Based on these findings it appeared that the fosmid modifications were results of insertions of chromosomal DNA from the donor into the fosmids. We suspected that the inserted chromosomal DNA was located near the integrated RP4, and that the insertions do occur as a consequence of co-activation of the chromosomally located oriT element (within RP4) during conjugation. As the fosmid vector used (pRS44) contains several elements from the RK2 plasmid, including oriV, oriT and parDE, it seemed possible that homologous recombination may occur between fosmid- and RP4 sequences. Correspondingly, the Tn7-sequences could possibly originate from the Tn7-transposon inserted into the kanamycin resistance gene of the integrated RP4, alternatively partly due to Tn7 transposition. As *E. coli* S17-1 is *oriT*+, homologous recombination should not occur within this donor host. However, it appeared fully feasible that the fosmid and DNA mobilized from the chromosomally located oriT could be co-transferred to the same recipient cell, and that homologous recombination could occur between these single-stranded molecules, given that the recipient was *oriT*-. Such a hypothesis may explain how DNA fragments originating from the *E. coli* chromosome are integrated into the fosmid clones.

**Construction of a new mobilization system for transfer of any plasmid containing oriT**

Given that the observed modification problems occur as a result of DNA-mobilization from oriT in the *E. coli* S17-1 chromosome, inactivation of oriT within the donor strain should eliminate the problem as this would block mobilization of the chromosomal DNA (a view also described by Babic and co-authors [12]). However, we also sought to eliminate the possibility of Mu genome mobilization as well as other reported *E. coli* S17-1/SM10-related problems, and in addition we wanted to circumvent the restriction of using one particular strain as donor. A plasmid-based mobilization system that provides the tra-gene products for mobilization of oriTRK2-containing vectors, which itself does not contain an intact oriT, could solve these issues. The most obvious strategy to achieve this appeared to be the substitution of the replication functions in an oriT-inactivated version of RK2 with another replication system. To avoid incompatibility problems with the plasmids to be transferred, the replicon of this new plasmid should satisfy three criteria: (i) it should not belong to the...
incompatibility groups of those plasmids that are heavily used in conjugal gene transfer experiments in bacteria, (ii) it should stably maintain the large regions from RK2 necessary to ensure intact Tra functions, and (iii) the new replicon should also be able to replicate in various bacteria (pBBR1 based plasmids are known to replicate in *Alcaligenes eutrophus*, *Bartonella bacilliformis*, *Bordetella spp.*, *Brucella spp.*, *Caulobacter crescentus*, *E. coli*, *Gluconacetobacter xylinus*, *Paracoccus denitrificans*, *Pseudomonas fluorescens*, *P. putida*, *Rhizobium meliloti*, *Rhodobacter sphaeroides*, *Salmonella typhimurium*, *Vibrio cholerae*, *X. campestris*). For this purpose the pBBR1 replicon was chosen which satisfies all the criteria listed above [18,19].

In order to make precise modifications of the large-sized RK2 plasmid (60 kb), a system was developed that allows for homologous recombination between ligated fragments within a suicide plasmid (pTAl0) and the target region of RK2, with subsequent selection of the altered RK2-derivatives (see Materials and Methods). This system was used to make two deletions within the RK2 plasmid, resulting in inactivation of the *oriT* site as well as the kanamycin resistance gene, generating the plasmid pTA19. Further, a fragment in pTA19 containing the replication origin, *oriV*, and the ampicillin and tetracycline resistance genes was replaced with a fragment containing the pBBR1 replicon and the gentamycin resistance gene (giving pTA-Mob, Figure 2).

Plasmids up to 220 kb in size were successfully transformed into *E. coli* DH10B/pTA-Mob cells, demonstrating that very large plasmids are maintained together with the relatively large-sized pTA-Mob. Next, *oriT*-containing plasmids were conjugated from *E. coli* DH10B/pTA-Mob cells using *P. fluorescens* as recipient, and the relatively easy attainment of transconjugants confirmed that the RK2 Tra functions were still intact. To test the system with regard to the described problem concerning modifications of conjugatively transferred plasmids, fosmid clones 37 and 83 were analysed after mobilization from *E. coli* DH10B/pTA-Mob.

Plasmids from 11 different *E. coli* analysed after mobilization from conjugatively transferred plasmids, fosmid clones 37 and 83 were all intact with respect to the described problem concerning modifications of *oriT* containing plasmids; *parABCD*, stabilization region encoding the gene products ParA, B, C, D and E; Ctl, central control operon of RK2 [6].

**Figure 2. Map of the mobilization helper plasmid pTA-Mob with relevant regions depicted.** Gm, gentamycin resistance gene; rep, pBBR1 replication protein gene; ori; pBBR1 replication origin; (trfA), replication initiation protein gene from the RK2 replicon, this replicon is not active due to lack of RK2 replication origin *oriV*, Tra1 and Tra2, regions containing the *tra* genes necessary for conjugal transfer of *oriT* containing plasmids; *parABCD*, stabilization region encoding the gene products ParA, B, C, D and E; Ctl, central control operon of RK2 [6].

**References**

1. Liu P, Balasubramaniamb S (2012) Opportunistic routing through conjugation in bacteria communication nanonetwork. Nano Commun Netw 3: 36–45.

2. Marraffini LA, Sontheimer EJ (2010). CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nat Rev Genet 11: 181–190.

**Acknowledgments**

We thank Rannveig Dahlrud for her help with some of the conjugal matings.

**Author Contributions**

Conceived and designed the experiments: TAS RL KFD SV. Performed the experiments: TAS RL KFD ML. Analyzed the data: TAS KFD SV. Contributed reagents/materials/analysis tools: TAS RL KFD ML. Wrote the paper: TAS RL SV.
3. Grohmann E, Muth G, Espinosa M (2003) Conjugative plasmid transfer in gram-positive bacteria. Microbiol. Mol. Biol. Rev. 67: 277–301.

4. Simon R, Priefer U, Puhler A (1983) A broad host range mobilization system for in vivo genetic engineering - transposon mutagenesis in gram-negative bacteria. Bio-Technol 1: 794–791.

5. Burkardt HJ, Riess G, Puhler A (1979) Relationship of group P1 plasmids revealed by heteroduplex experiments. RP1, RP4, R68 and RK2 are identical. J. Gen Microbiol 114: 341–346.

6. Pansegrau W, Lank E, Barth PT, Figurski DH, Guiney DG, et al. (1994) Complete nucleotide sequence of Birmingham IncP alpha plasmids. Compilation and comparative analysis. J. Mol Biol 239: 625–663.

7. Brantaet T, Lalé R, Valla S (2009) Positively regulated bacterial expression systems. Microb. Biotechnol 2:15–30.

8. Brautaset T, Lalé R, Valla S (2009) Positively regulated bacterial expression systems. Microb. Biotechnol 2:15–30.

9. Herrero MV, de Lorenzo V, Timmis KN (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol 172: 6557–6567.

10. Wiater LA, Marra A, Shuman HA (1994) Escherichia coli F plasmid transfers to and replicates within Legionella pneumophila: an alternative to using an RP4-based system for gene delivery. Plasmid 32: 280–294.

11. Ferrière L, Hémony G, Nham T, Guérout AM, Mazel D, et al. (2010) Silent mischief: bacteriophage Mu inserts conserved products of Escherichia coli random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugal machinery. J. Bacteriol 24: 6418–27.

12. Babic A, Guérout AM, Mazel D (2008) Construction of an improved RP4 (RK2)-based conjugal system. Res Microbiol 159: 545–549.

13. Priefer UB, Simon R, Puhler A (1985) Extension of the host range of Escherichia coli vectors by incorporation of RSF1010 replication and mobilization functions. J. Bacteriol 163: 324–330.

14. Wexler M, Bond PL, Richardson DJ, Johnston AW (2005) A wide host-range metagenomic library from a waste water treatment plant yields a novel alcohol/aldehyde dehydrogenase. Environ Microbiol 7: 1917–1926.

15. Askvik T, Degnes KF, Dahlstrud R, Schmidt F, Dam R, et al. (2009) A plasmid RK2-based broad-host-range cloning vector useful for transfer of metagenomic libraries to a variety of bacterial species. FEMS Microbiol Lett 286: 149–158.

16. Sambrook J, Russell DW (2001) Molecular cloning, a laboratory manual. New York: Cold Spring Harbor Laboratory Press.

17. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, et al. (1993) Four new derivatives of the broad-host-range cloning vector pBHR1MCS, carrying different antibiotic-resistance cassettes. Gene 166: 173–176.

18. Lefebre MD, Valvano MA (2002) Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in Burkholderia cepacia complex isolates. Appl. Environ. Microbiol 68: 5956–5964.

19. Valla S, Frydenlund K, Goucheron DH, Haugan K, Johannsen B, et al. (1992). Development of a gene transfer system for curing of plasmids in the marine fish pathogen Flavobacterium salmonica. Appl. Environ. Microbiol 58: 1980–1985.

20. de Lorenzo V, Cases I, Herrero M, Timmis KN (1993) Early and late responses of TOL promoters to pathway inducers: identification of postexponential promoters in Pseudomonas putida with lacZ-tet bicistronic reporters. J. Bacteriol 175: 6902–6907.

21. Hotte B, Rath-Arnold I, Puhler A, Simon R (1990) Cloning and analysis of a 35.3-kilobase DNA region involved in exopolysaccharide production by Xanthomonas campestris pv. campestris. J. Bacteriol 172: 2004–2007.