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A new family of conditional replicating plasmids and their cognate *Escherichia coli* host strains

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Abstract

We constructed a family of conditionally replicating plasmids, the pTX1 family, which are based on the IncPα *oriV* origin of replication that is dependent on the *trfA*-encoded protein. We constructed several *Escherichia coli* derivatives expressing *trfA* from different chromosomal loci, which can be transduced by phage P1 to any *E. coli* strain. The pTX1 plasmids also carry the $oriT_{RP4}$ origin of transfer, and can be conjugated to *E. coli*, *Vibrio cholerae* and likely to a broad range of bacteria from the commonly used donor strains SM10 and S17-1, which sustain replication of the plasmids through the *trfA* gene carried by their integrated RP4. If TrfA is not provided in trans, these plasmids behave as suicide vectors. As such they can be used as a platform for a variety of applications such as those developed on the popular conditionally replicating plasmids carrying the $oriV_{R6K\gamma}$ origin of replication that is controlled by the Π protein. Their ability to be used as efficient suicide vectors for gene disruption in *V. cholerae* has been demonstrated.

Keywords: Suicide plasmids; Conjugation; Mutagenesis

1. Introduction

Allelic replacement and transposon mutagenesis are powerful techniques that form the basis of many physiological and genetic studies of bacteria. However, these approaches are still difficult to achieve in numerous species. Indeed, only a few bacterial species are naturally competent for DNA transformation, while artificial transformation is inoperative, or inefficient, for many other species. In numerous cases the limitations for exogenous DNA delivery have been overcome by using, as a first step, conjugation with broad host range plasmids, and several systems have been described based upon the IncPα plasmid RP4 (RK2) transfer functions (see for example [22]). The second step, replacement or integration, is in most cases achieved through the use of a non-replicative DNA molecule; the most popular system for Gram-negative species is the one using conditionally replicative R6K plasmid derivatives, such as pGP704 [18]. The R6K plasmid is a member of a large group of replicons (IncX) whose replication depends on the binding of an initiator protein to specific nucleotide sequence repeats called iterons [8,11,12]. R6K replication is dependent on the binding of the *pir* encoded Π protein, and trans-complementation of a *pir*-dependent plasmid derivative by a Π protein expressed from another replicon can be performed [13]. From this seminal observation several plasmids carrying the R6Kγ origin of replication that can only be replicated in strains expressing *pir* have been constructed. When these plasmids also carry a RP4 (or RK2) transfer origin, they can be transferred to various bacterial cells through the broad-host range conjugation system of RP4. As these plasmids behave as suicide vectors in pir^- recipients, they have been successfully used to create mutants through gene disruption by insertion [18] or transposon mutagenesis [9]. A wide range of Gram-negative bacteria can be engineered with such tools and most of the proteobacteria can be used as recipients for conjugation (see for example [6,7,9,16–18,23]). Several counterselectable markers have been described [19] and some of them have been successfully used in R6K–$oriT_{RP4}$ derivatives for the positive selection of replaced alleles [7,23].

However, a limitation of this technology resides in the fact that all derivatives of these plasmids carry two regions

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of identity totalling at least 400 bp: a R6Kγ origin of replication and an RP4 oriT. These regions of identity can be the source of recurrent problems when further insertions using the same technique are attempted in strains that already carry such a derivative integrated into their genome, as mis-integrations of the next suicide vector could occur through homologous recombination between the identical $oriV_{R6K\gamma}$ or $oriT_{RP4}$ regions they share instead of between the vector and the targeted chromosomal loci.

In order to circumvent these problems we have developed a broad host range conjugative platform differing from the IncP RP4 family (G. Demarre and D. Mazel, in preparation) and a family of conditional replicating plasmids, the pTX family, which do not rely on R6Kγ. These plasmids are based on the IncPα *oriV* origin of replication for which replication is dependent on the *trfA* encoded protein [3,14]. Four different derivatives expressing different antibiotic resistance markers and carrying the RP4 *oriT* have been built. We constructed three *E. coli* derivatives harboring the *trfA* gene integrated in their chromosome, which support the replication of these plasmids and allow their production. We also demonstrated that the pTX1 derivatives can be replicated and transferred by conjugation from the commonly used S17-1 and SM10 *E. coli* strains, which carry a RP4 immobilized in their chromosome, to different types of bacterial species.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Tables 1 and 2, respectively. *E. coli* strains were grown in Luria–Bertani (LB) or, when specified, Mueller–Hinton (MH) broth at 37 °C, or 30 °C when grown to perform allelic exchange experiments. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg/ml, chloramphenicol (Cm), 25 μg/ml, gentamicin (Gm), 10 μg/ml, kanamycin (Km), 25 μg/ml, streptomycin (Sm) 50 μg/ml, spectinomycin (Sp) 50 μg/ml, and thymidine (dT) was supplemented when necessary to a final concentration of 0.3 mM. Methionine (Met) was added at 0.01% final concentration. Primers were obtained from Proligo (France).

2.2. Polymerase chain reaction (PCR) procedures

PCR performed for plasmid assembly was done in 50 μl volumes using the *Pfu* DNA polymerase (Promega) following the manufacturer's instructions. Other PCR reactions were performed in 50 μl volumes using PCR Reddy Mix (Abgene, UK) according to the manufacturer's instructions. The primers for PCR are listed in Table 3. Conditions for amplification were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60–360 s (as a function of the expected product size from 1 to 6 kb).

2.3. Construction of the thyAΔ::aac3′.IV-trfA E. coli strain ω135

The *E. coli thyA* locus and its neighborhood were amplified from the *E. coli* MG1655 genome using primers Thy1 + Thy2 (Table 3). After *Eco*RI digestion, the generated 1628-bp PCR fragment, encompassing *thyA*, the upstream 503 bp and the downstream 351 bp, was cloned at the *Eco*RI site of pNot218, generating plasmid p1003 (Table 2). Plasmid p1539, a p1003 derivative in which the *thyA* gene was deleted from the ATG to the stop codon and substituted by an *Mfe*I site (*thyA*Δ), was constructed by inverse PCR from p1003 using primers Thy3 and Thy5, followed by digestion with *Mfe*I and self ligation.

Table 1
Bacterial strains used in this study

E. coli strains	Description/relevant characteristics[a]	Reference
MG 1655	*E. coli* K12 wild type	Laboratory collection
DH5α	*supE44* Δ*lacU169* (Φ*80lacZ*Δ*M15*)Δ*argF hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) (F′ *lacZ*Δ*M15 lacI*^{q} *traD36 proA*$^{+}$, *proB*$^{+}$)	Laboratory collection
BW19610	(*lacJZYA*)(*lac*)*X74* Δ*uidA*::*pir-116 recA1* Δ*phoA532* Δ(*phnc*?*DEFGHIJKLMNOIP*)*33-30*	[17]
SM10	RP4-2-Tc::Mu *recA* [Km^{R}]	[22]
S17-1	RP4-2-Tc::Mu *aph*::Tn*7 recA* [Sm^{R}]	[22]
β2163	MG1655 *dapA*Δ::*erm* RP4-2-Tc::Mu [Km^{R}]	D. Mazel, unpublished
DH5α(p1437)	DH5α (pSU38::trfA)	This work
DH5α(pKOBEG)	DH5α (pSC101^{ts}::P$_{bad}$ redγβα)	[4]
TG1(pKOBEG)	TG1 (pSC101^{ts}::P$_{bad}$ redγβα)	[4]
ω135	DH5α *thyA*Δ::*aac3′.IV-trfA* [Gm^{R}]	This work
ω170	DH5α *metA*Δ::*aac3′.IV-trfA* [Gm^{R}]	This work
ω182	TG1 *metA*Δ::*cat-trfA* [Cm^{R}]	This work

[a] Abbreviations used for antibiotic resistance (^{R}): Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Sm, streptomycin, Sp, spectinomycin. Concentrations used to select for antibiotic resistance are given in the text.

Table 2
Plasmids used in this study

Plasmid	Description[a]	Reference
pJB7	Derivative of pFF1 (RK2 minimal replicon), Ap^R	[2]
pJB3	Derivative of pJB7 carrying *bla*, Ap^R	[2]
pJB3Δ	Self ligation of the largest *Pvu*II fragment of pJB3, Ap^R	This study
p1509	*Pvu*II–*Pvu*II MCS polylinker and *lacZ*α fragment from pUC18 cloned into pJB3Δ (*Pvu*II), Ap^R	This study
pTX1a	p1509 deleted of the duplicated 89 bp	This study
pTX1k	Identical to pTX1a except *bla* was replaced by *aph*, Km^R	This study
pTX1c	Identical to pTX1a except *bla* was replaced by *cat*, Cm^R	This study
pTX1g	Identical to pTX1a except *bla* was replaced by *aac3′-IV*, Gm^R	This study
pSU38	$oriV_{P15A}$, Km^R	[1]
p1437	pSU38::*trfA*; pJB7 *Pst*I–*Pvu*II fragment containing the *trfA* gene cloned in pSU38 (*Pst*I–*Sma*I), Km^R	This study
pSB118	pUC18 with a second *lacZ*α in-frame *Eco*RI site 3 nt downstream of the unique *Hin*dIII, Ap^R	J. Bouvier and P. Stragier, unpublished
p1512	pSB118::*trfA*; *Kpn*I–*Pst*I fragment from p1437 cloned in pSB118 (*Kpn*I–*Pst*I), Ap^R	This study
p1521	pSB118::*aac3′.IV-trfA*; *aac3′.IV Kpn*I–*Kpn*I PCR fragment (AprKp1, AprKp2) of cartridge in p1512 (*Kpn*I–*Kpn*I), Km^R Gm^R	This study
pNot218	pTZ18R containing two *lacZ*α in-frame *Not*I sites (AF480833), Ap^R	[20]
p1003	pNot218::*thyA*; *Eco*RI–*Eco*RI PCR product (Thy1, Thy2) of *ThyA* gene into pNot218 (*Eco*RI), Ap^R	This study
p1539	pNot218::*thyA*Δ; (Thy3 + Thy5) inverse PCR on p1003, digested at the added *Mfe*I sites and self-ligated, Ap^R	This study
p1541	pNot218::*thyA*Δ::(*aac3′.IV-trfA*); *Eco*RI–*Eco*RI *aac3′.IV-trfA* fragment from p1521 cloned in *Mfe*I digested p1539, Ap^R Gm^R	This study
pSW25	$oriV_{R6K}$, Sp^R Sm^R	D. Mazel, unpublished
p1559	pSW25::*thyA*Δ::(*aac3′.IV-trfA*); *Eco*RI–*Eco*RI *thyA*Δ::(*aac3′.IV-trfA*) fragment from p1541 in pSW25 (*Eco*RI), Gm^R Sp^R Sm^R	This study
p2254	pSB118::*cat-trfA*; *Kpn*I–*Kpn*I *cat* gene PCR fragment (CatKp1 + CatKp2) in p1512(*Kpn*I), Ap^R Cm^R	
p2279	pNot218::Δ*thyA*::(*cat-trfA*); *Eco*RI–*Eco*RI *cat-trfA* fragment from p2254 in p1539 (*Mfe*I), Ap^R Cm^R	This study
p2286	pSW25::Δ*thyA*::(*cat-trfA*); *Eco*RI–*Eco*RI Δ*thyA*::(*cat-trfA*) fragment from p2279 into pSW25 (*Eco*RI), Ap^R Cm^R	This study

[a] Abbreviation used for antibiotic resistances(^{R}): Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Sm, streptomycin, Sp, spectinomycin. Concentrations used to select for antibiotic resistances are given in the text.

Two different *thyA*Δ alleles harboring *trfA* and an antibiotic resistance marker were constructed as follows. We first constructed a plasmid expressing *trfA* by subcloning the *Pst*I–*Pvu*II fragment carrying *trfA* from pJB7 [2], in the p15A derivative pSU38 [1] that was digested by *Pst*I–*Sma*I, creating plasmid p1437. The p1437 *Kpn*I/*Pst*I fragment containing the *trfA* gene was then cloned in pSB118, leading to p1512. The *aac3′.IV-trfA* and *cat-trfA* were built by cloning, at the unique *Kpn*I site of p1512, upstream of the *trfA* gene, the *aac3′.IV* or *cat* genes after amplification with primers (AprKp1 + AprKp2) or (CatKp1 + CatKp2), respectively, and digestion with *Kpn*I. One plasmid of each cloning, p1521 and p2254, respectively (Table 2), carrying the resistance marker in the same orientation as *trfA*, was further used.

Plasmid p1541 was assembled by ligation of the *aac3′.IV-trfA Eco*RI fragment from p1521 at the *Mfe*I site introduced between the *thyA* 5′ and *thyA* 3′ segments of p1539. Plasmid p2279 was assembled by ligation of the *cat-trfA Eco*RI fragment from p2254, at the *Mfe*I of p1539. The *thyA*Δ alleles carried by p1541 and p2279 are hereafter referred to as *thyA*Δ::aac3′*.IV-trfA* and *thyA*Δ::*cat-trfA*, respectively. Both p1541 and p2279 *thyA*Δ alleles were then transferred to pSW25, a conditional replicating R6K derivative, leading to p1559 and p2286, respectively.

Construction of the *E. coli* ω135, a *thyA*Δ::aac3′*.IV-trfA* derivative of DH5α, was performed following the allele replacement procedure described by Chaveroche and collaborators [4]. Briefly, once prepared, electrocompetent DH5α (pKOBEG) cells expressing the λ *red* genes were transformed with 1 μg of the PCR product obtained using primers Thy1 + Thy2 on p1559 as template, and plated on the appropriate selective plates, MH dT Gm.

2.4. *Construction of the* metAΔ::aac3′.IV-trfA *and* metAΔ::cat-trfA E. coli *strains ω170 and ω182*

The *metA*Δ alleles were assembled in vitro through a two-step PCR construction method. We independently amplified from *E. coli* MG1655 genomic DNA the region starting 103 bp upstream of *metA* and ending at codon 109 using primers MetA1 and MetA2*, and the region starting at

Table 3
Oligonucleotides used in this study

Oligonucleotide	Sequence	Restriction site[a]
atrcA1	CAATAATATTGAAAAAGGAAGAGTATGCAATACGAATGGCGAAAAGCCGAGC	
atrcA1C	GCTCGGCTTTTCGCCATTCGTATTGCATACTCTTCCTTTTTCAATATTATTG	
atrcA2	GCTAGACAATTGCTGTCAGACCAAGTTTACTCATAT	*Mfe*I
atrcA3	ACGGTACAATTGTCAGCCAATCGACTGGCGAGCG	*Mfe*I
ktrcA1	CAATAATATTGAAAAAGGAAGAGTATGATTGAACAAGATGGATTGCAC	
ktrcA1C	GTGCAATCCATCTTGTTCAATCATACTCTTCCTTTTTCAATATTATTG	
ktrcA2	GCTAGACTCGAGCTGTCAGACCAAGTTTACTCATAT	*Xho*I
ktrcA3	ACGGTACTCGAGTCAGAAGAACTCGTCAAGAAGG	*Xho*I
pTXC.1	CAATAATATTGAAAAAGGAAGAGTATGGAGAAAAAAATCACTGGATATACCAC	
pTXC1.1C	GTGGTATATCCAGTGATTTTTTTCTCCATACTCTTCCTTTTTCAATATTATTG	
pTXC.3	ACGGTACTCGAGTTACGCCCCGCCCTGCCACTCAT	*Xho*I
DelDu.1	GATATCGAGATCTGAATTAATTCTTGAAGACGAAAGGG	*Bgl*II
DelDu.2	CGCCAGCAGATCTGCATGTTCTTT	*Bgl*II
AprKp1	GGGGTACCTTCATGTGCAGCTCCATCAG	*Kpn*I
AprKp2	CCGGTACCGGGCATTGAGCGTCAGCA	*Kpn*I
Thy1	TAAGAATTCCGTTTGGTCTTGGTGCCG	
Thy2	CGGGAATTCACGTAAATAGAGCAAATAGTCC	
Thy3	AGATCTCAATTGGCTATCTAATTACGAAACATCC	*Mfe*I
Thy5	AATGATCAATTGTCAGGAAACGTGTTGCTGTGGG	*Mfe*I
CatKp1	TATGGTACCGCGCCGAATAAATACCTGTGA	*Kpn*I
CatKp2	TTCGGTACCGCGCAGACCAAAACGATCTCA	*Kpn*I
MetA1	GAATTCTCACCTTCAACATGCAGGCTCG	*Eco*RI
MetA2*	GAAACGTGTTGCTGTGGGCTGCCAATTGGGCGCACCAGTTACAATCAAACCGTC	
MetA3*	TCTAATTACGAAACATCCTGCCAATTGGGGATGCATATCTGTTTGCCAGTAAAG	
MetA4	GAATTCTATTCACCTGCTGAGGTACGTTTCGG	*Eco*RI
TTA1	TGCGCCCAATTGGCAGCCCACAGCAACACGTTTCCTGA	*Mfe*I
TTA2	ATGCATCCCCAATTGGCAGGATGTTTCGTAATTAGATAGC	*Mfe*I
M13 reverse	AGCGGATAACAATTTCACACAGGA	
VCR1	GTCCCTCTTGAGGCGTTTGTTA	

[a] Oligonucleotide restriction endonuclease recognition sites used for plasmid construction. See Table 2.

codon 217 of *metA* and ending 98 bp downstream of the *metA* stop codon (310) using primers MetA3* and MetA4. We also independently amplified the *aac3′.IV-trfA* and *cat-trfA* fragment using primers TTA1 and TTA2 from p1559 and p2286. The TTA1 and TTA2 primers were respectively designed from the sequence just upstream and just downstream from the *Mfe*I site of the *thyA*Δ allele, facing each other in order to allow the amplification of any sequence cloned at this site. The primers MetA2* and MetA3* also included a sequence complementary to the TTA1 and TTA2, respectively.

After gel purification of all four different PCR products, two independent assemblies were set up by mixing together 100 ng of three PCR products: those from (MetA1 + MetA2*), (MetA3* + MetA4), and the (TTA1 + TTA2) product from either p1559 or p2286. The final step was carried out by PCR with the most external primers metA1 and metA4. The expected *metA*Δ::*aac3′.IV-trfA* and *metA*Δ::*cat-trfA* products were then gel-purified and 1 µg of each was used for the allelic exchange procedure as described for the ω135 construction and selection on LB + Gm or LB + Cm. ω170, a DH5α *metA*Δ::*aac*3′.*IV-trfA* derivative and ω182, a TG1 *metA*Δ::*cat-trfA* derivative, were further studied and used (Table 1).

2.5. Conjugations

The different conjugation experiments were done by the filter mating procedure as described by Timmis and collaborators [9], with a recipient/donor ratio of 10. The transfer of pTX1a was tested from SM10 to ω135 using a 1 h mating time and transconjugants selected on LB Gm Ap. Conjugation frequencies were calculated as the number of [Gm^R Ap^R] colonies compared to the number of donors ([Km^R]). The presence of pTX1a in a sample of 6 transconjugants was verified by plasmid minipreparation. Suicide conjugation from SM10 to an Sm^R derivative of *Vibrio cholerae* N16961 was performed in overnight filter mating, using pTX1a::*catB9*, a pTX1a derivative carrying the *catB9* gene found in the *V. cholerae* N16961 superintegron. pTX1a::*catB9* was constructed by cloning *catB9* from the pBADVchCAT [21] into pTX1a that was digested with *Eco*RI and *Pst*I. *V. cholerae* [Ap^R Sm^R] transconjugants were obtained and the conjugation-recombination frequency calculated as the number of [Ap^R Sm^R] recipients from the total number of recipient ([Sm^R]). The chromosomal integration of pTX1a::*catB9* through homologous recombination with the chromosomal *catB9* copy was verified by PCR analysis using primers M13 reverse and VCR1 [15].

3. Results and discussion

3.1. pTX1a construction

Since our objective was to produce plasmids for which replication was dependent on TrfA provided in trans, we first constructed a plasmid expressing the *trfA* gene by subcloning the *Pst*I–*Pvu*II fragment carrying *trfA* from pJB7, in the p15A derivative pSU38 digested by *Pst*I–*Sma*I, leading to plasmid p1437. We then constructed an $oriV_{RP4}$ plasmid deleted of the *trfA* gene, pJB3Δ, from the plasmid pJB3, an RK2 autoreplicative derivative described by Blatny and collaborators [2]. This was achieved through digestion of pJB3 with *Pvu*II, self-ligation of the 2.6-kb fragment carrying *bla*, $oriV_{RK2}$ and *oriT* and transformation of both DH5α and DH5α (p1437) expressing *trfA* in trans. As expected, no Ap^R clones were obtained in the DH5α transformation attempt, while many Ap^R clones were obtained in DH5α (p1437). Analysis showed that, in addition to the p1437, these Ap^R clones carried the expected 2.6 kb long plasmid pJB3Δ, demonstrating that the replication can be sustained by *trfA* in trans. Construction of the conditional replicating plasmid pTX1a was achieved by ligating the *Pvu*II fragment carrying the multiple cloning sites and lacZα from pNot218 to pJB3Δ digested by *Pvu*II, and transformation of DH5α carrying p1437. One plasmid, p1509, showing the expected size and restriction map was chosen for further constructions. We noticed that pJB3 carried an inverted duplication of 89 bp corresponding to a portion of the *lacZα* gene, located outside of the *Pvu*II fragment carrying the *lacZα* and polylinker region. This inverted duplication was also by essence present in our plasmid p1509 and proved problematic as it impeded the use of the classical M13 forward primers, which hybridized to the duplicated segment. We then deleted the duplicated copy located outside of the polylinker *Pvu*II fragment using inverse PCR with primers DelDu.1 and DelDu.2, followed by digestion with *Bgl*II, self ligation and transformation of strain ω135 described below. One plasmid, pTX1a, showing all expected properties (expected size and restriction map), was chosen for further constructions.

3.2. pTX1k, pTX1c and pTX1g constructions

Three additional pTX1 derivatives: pTX1k [Km^R], pTX1c [Cm^R] and pTX1g [Gm^R], were constructed using pTX1a as template. These three pTX1 derivatives were assembled following the principle depicted in Fig. 1 for the construction of pTX1k. In each case, we designed primers in order to amplify pTX1a devoid of the *bla* gene from the ATG to the TAA codons. We designed the primers on the ATG side in such a way that they carried the sequence of the first 8 codons of the resistance gene chosen to substitute for *bla*: *aph*, *cat*, or *aac3′-IV*. The TAA3′ primers were also extended in order to introduce a unique restriction site downstream of the resistance marker stop codon, *Xho*I for *aph* (pTX1k) and *cat* (pTX1c), *Mfe*I for *aac3′-IV* (pTX1g). The different resistance genes were amplified using a 5′ primer extended in 5′ by the 24 nt region covering the *bla* RBS up to the ATG in pTX1a. The 3′ primers of each alternative resistance genes were extended in order to introduce the same unique restriction site as that used to amplify the pTX1 core (see Fig. 1 and Table 3). pTX1k was finally assembled by mixing 100 ng of the (ktrcA2 + ktrcA1C) pTX1 core PCR product with 100 ng of the (ktrcA1 + ktrcA3) *aph* PCR product and by performing a PCR amplification using primers ktrcA2 and ktrcA3. The expected product was then gel-purified, digested with *Xho*I and self-ligated to create pTX1k. pTX1k was introduced by heat-shock into the *trfA* expressing strain ω135 described below. pTX1c was created by the same strategy using the products from the (pTXC.1C + ktrcA2) and (pTXC.3 + pTXC.1) PCRs and a final assembly using primers pTX1.1 and pTX1.3. pTX1g was built using the products from the (atrcA1C + atrcA2) and (atrcA3 + atrcA1) PCRs in a final step of assembly using primers atrcA1 and atrcA2 (Fig. 1 and Table 3) and transformation of the strain ω182 described below.

3.3. Construction of a set of E. coli strains sustaining pTX1 replication

We constructed three *E. coli* derivatives carrying a chromosomal *trfA* gene, in order to facilitate the manipulations of the different pTX1 derivatives. We inserted *trfA* in two different chromosomal loci, *thyA* and *metA*. *thyA* encodes thymidylate synthetase, and in consequence *thyA* mutants require thymidine to grow. *metA* codes for the homoserine O-succinyltransferase and *metA* mutants require methionine to grow. We built two *thyA*Δ alleles, *thyA*Δ::*aac3′.IV-trfA* and *thyA*Δ::*cat-trfA*, respectively, [Gm^R] and [Cm^R], and two *metA*Δ alleles: *metA*Δ::*aac3′.IV-trfA* and *metA*Δ::*cat-trfA*, respectively, [Gm^R] and [Cm^R]. The wild-type *thyA* was substituted by the *thyA*Δ::*aac3′.IV-trfA* allele in the chromosome of DH5α to provide strain ω135. Another DH5α derivative carrying the *metA*Δ::*aac3′.IV-trfA* allele on the chromosome, ω170 was also constructed. Finally, ω182, a TG1 derivative carrying a chromosomal *metA*Δ::*cat-trfA* allele was built (Table 1).

We then tested these three strains and also the commonly used *E. coli* strains SM10 and S17-1, which both carry a *trfA* gene encoded in their chromosomally integrated RP4. ω135, ω170, ω182 and SM10 were able to replicate the different pTX1s. Curiously, we found that S17-1 was able to replicate the pTX1a, pTX1g and pTX1k, but not pTX1c; the cause of this last observation is unknown.

3.4. Transfer of pTX1a from E. coli to E. coli

We tested the ability of the pTX1a to be transferred using its $oriT_{RK2}$ from SM10 using the RP4 transfer functions encoded by the integrated RP4. Conjugation by the filter

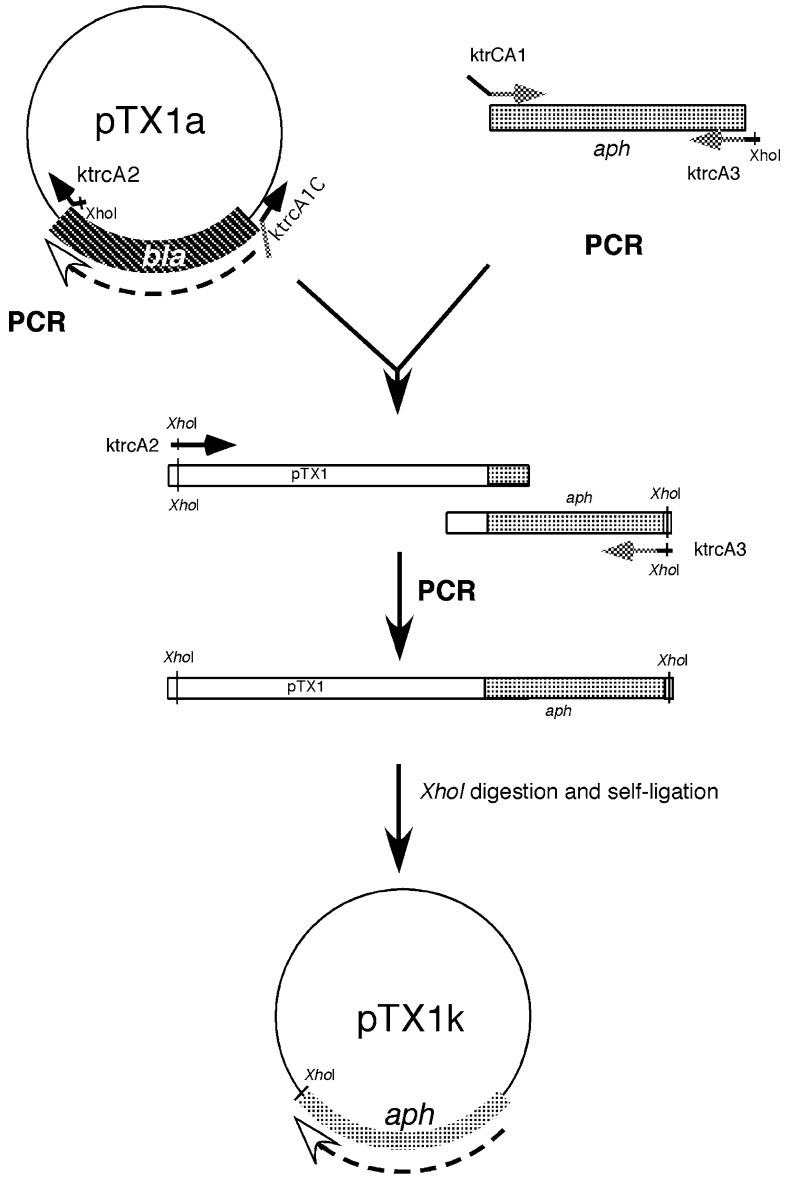

Fig. 1. Schematic representation of the resistance gene substitution process used in construction of the different pTX1 derivatives. PCR primers are shown as solid black arrows. Relevant restriction sites are also indicated. Resistance gene orientations are indicated by patterned arrows.

mating technique was tested from SM10 to ω135 and transfer of pTX1a to ω135 was observed to occur at frequency of about 1 (9.6×10^{-1} on average).

3.5. *Transfer of pTX1a from* E. coli *to* V. cholerae

We also tested the ability of the pTX1a to be transferred from SM10 as suicide vector to perform chromosomal integration through homologous recombination between a pTX1a carried insert and the corresponding target in the chromosome of a chosen bacteria. We tested this capability for *V. cholerae* using the *catB9* cassette of strain N16961 [21] as a target for recombination and integration in the *V. cholerae* chromosome 2. Ap^R transconjugants of *V. cholerae* were obtained at a frequency of 7×10^{-7}, on the same order as that obtained (10^{-7} on average) with the commonly used pGP704 as delivery vector and SM10-λpir as the donor strain. The presence of the pTX1a::*catB9* integrated at the proper site in the chromosome was verified by PCR with the appropriate primers (data not shown).

3.6. *Conclusions*

We have developed a family of conditional replicating plasmids, the pTX1 family, which do not rely on R6Kγ *oriV*. These plasmids, are based on the IncPα *oriV* origin of replication for which replication is dependent on the *trfA* encoded protein. We also constructed several *E. coli* derivatives expressing *trfA* from two chromosomal loci. These alleles can be transduced by P1 to any *recA*$^+$ strain. A set of IncPα *oriV* plasmids for which replication was made conditional through control of the *trfA* expression by a regu-

lated promoter had been previously developed by Valla and collaborators [10]. However, in their publication these authors mentioned that the control was variable with the *E. coli* host strain and, for example, was inefficient in MC4100. We chose to develop a system in which TrfA was provided in trans in order to circumvent such limitation. In mutant construction attempts through single recombination, this separation also prevents the appearance of false-positives in non-permissive conditions, which would be due to the selection of mutants of the controlled promoter and which would be phenotypically indiscernible from integrated plasmid.

The pTX1 plasmids also carry the $oriT_{RP4}$ and can be transferred from the commonly used donor strains SM10 and S17-1, which carry an integrated RP4, to *E. coli*, *V. cholerae* and very likely to a broad range of bacteria. If TrfA is not provided in trans, the pTX1 plasmids will behave as suicide vectors. As such they can be used as platform for a variety of applications, such as the constructions of mini-*Tn* delivery vehicles [9], for mutant constructions through single recombination (see for example [16]), or to carry the resistance markers for allelic exchange techniques based on PCR product transformation [4,5]. The pTX1 family also offers the possibility of co-maintaining, in the same strain, two conditionally replicating plasmids, for example, together with a *pir*-dependent plasmid such as pGP704 [18], as long as the strain carries both the *pir* and *trfA* genes in its chromosome. This is the case for strains SM10-λpir and S17-1-λpir, and we verified the compatibility of pGP704 and pTX1g in SM10 (not shown).

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