



# Plasmids containing the same origin of replication are useful tools to perform biotechnological studies in *Pseudomonas putida* U and in *E. coli* DH10B

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## Abstract

Plasmids containing the same origin of replication (pBBR1MCS-2 Km<sup>R</sup> and pBBR1MCS-3 Tc<sup>R</sup>) have been used to express simultaneous and independently different proteins in *P. putida* U and in *E. coli*. Thus, when *P. putida* was transformed with different genetic constructions made in the same plasmid (pBBR1MCS-3 Tc<sup>R</sup>), or with plasmids containing the same replication origin but with different antibiotic resistant genes (Km<sup>R</sup> and Tc<sup>R</sup>), they co-existed inside the same microbe. Furthermore, when *E. coli* DH10B was transformed with the plasmids recovered from the recombinant *P. putida* U, we noticed that all the bacteria isolated from single colonies are resistant to Km and Tc, suggesting that these plasmids were also present in *E. coli*. This observation facilitates the genetic manipulation of these strains (i.e. avoiding the use of different plasmids in double or multiple complementation experiments), and could be an interesting tool to approach many metabolic and biotechnological studies.

**Keywords:** Plasmid compatibility, Complementation, *P. putida*,  $\beta$ -oxidation

## Introduction

The transformation of bacteria with plasmids is a basic molecular process used in genetic engineering for over 40 years [1]. These genetic elements confer to the recipient microbe important metabolic and ecological advantages, as well as a remarkable biotechnological interest [2].

It has been well established that some bacteria may contain more than one plasmid co-residing in the same cell, and that they are transmitted to nascent daughter cells during cell division [3]. These plasmids, which although different, are maintained in the same bacterium, are called compatible plasmids. Conversely, in other cases, plasmids cannot co-exist in the same bacteria [1, 4]. Incompatible plasmids are for different reasons: (i) to the sharing of elements involved in the replication (usually the initiator protein which is often a limiting factor) or (ii) to interference phenomena involved on partitioning (regular distribution of the plasmid copy number in the recipient bacterium) [3, 4]. Although it is generally accepted that plasmids containing the same origin of replication are incompatible in the same bacterium, this item is subject to some controversy [5].

In this report, we presented evidences showing that plasmids containing a common replication origin co-exist in the same microbe, and that their use could be effective tools to achieve different biotechnological approaches.

## Materials and Methods

### Plasmids and bacterial strains

The plasmids used in all the experiments were pBBR1 derivatives. They belong to a family of broad-host-range cloning and expression vectors (pBBR1MCS-2 Km<sup>R</sup>; pBBR1MCS-3 Tc<sup>R</sup>; pBBR1MCS-4 Ap<sup>R</sup> and pBBR1MCS-5 Gm<sup>R</sup>) that replicate autonomously in *Pseudomonas* [6].

*Pseudomonas putida* U (PpU, Rf<sup>R</sup>, Colección Española de Cultivos Tipo, CECT 4848) was from our collection [7]. *P. putida*  $\Delta$ *fadBA* (PpU  $\Delta$ *fadBA*) is a poly-3-hydroxy-n-alkanoate (PHAs) over-producer mutant in which the *fadBA* genes, encoding the proteins FadB and FadA belonging to the main  $\beta$ -oxidation pathway, have been deleted [8]. This mutant, although slowly, grows in minimal media containing n-alkanoic acids as the sole carbon source, but it does not grow in media containing n-aryl-alkanoic acids. The analysis of these catabolic behavior revealed that in PpU  $\Delta$ *fadBA*, a different set of inducible  $\beta$ -oxidation enzymes replaces FadB and FadA in the pathway required for the assimilation of n-alkanoic acids. However, these enzymes are unable to degrade aryl-alkanoates, showing that FadBA are essential for the degradation of n-arylalkanoic acids [9].

The strains *Escherichia coli* DH10B [10] used for plasmid propagation, and *E. coli* pRK600 (Cm<sup>R</sup>) used as helper strain in triparental filter mating [11], were obtained from different laboratories [12].

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## Molecular biology studies

DNA manipulations were performed as indicated elsewhere [8, 12, 13]. Plasmids were extracted and purified using a GeneJET plasmid MiniprepKit (Thermo Scientific, Lithuania). The genes *fadA* and *fadB* were PCR-amplified from *P. putida* U DNA following the procedure described by Miñambres et al. [14].

## Culture conditions

Bacterial strains were cultured in slants containing LB medium (*E. coli*) or in a chemically defined medium (MM) [7] containing 4-hydroxy-phenylacetic acid (4-OH-PhAc, 10 mM) as the sole carbon source (*P. putida* U).

## Plasmid transformation

Bacteria (*PpU*, *PpU*  $\Delta$ *fadBA*, *E. coli* pRK600 -helper- and *E. coli* DH10B recombinants containing each of the plasmids, or the derived genetic constructions carrying the genes *fadB*, *fadA* or *fadBA*) were cultured on LB (at 30°C -*PpU*- or 37°C -*E. coli*-) for 8 h ( $Abs_{540}=2.0$ ). Cultures (1 ml) were collected, mixed and centrifuged (1 min at 13,000 rpm). The pellet was washed twice with 0.5 ml of LB, re-suspended in 50  $\mu$ l of LB, put on mating filters and incubated (30°C) on LB plates overnight. Bacteria were re-suspended from the filters in 1 ml of LB medium, diluted properly, and streaked on agar LB plates containing rifampicin, tetracycline and kanamycin ( $LB_{Km^R}Rf^R$  $Tc^R$ ). When required, a chemically defined medium containing 10 mM 6-phenylhexanoic acid as the sole carbon source ( $PhHKm^R$  $Rf^R$  $Tc^R$ ) was used.

When necessary, different antibiotics (rifampicin, 20  $\mu$ g ml<sup>-1</sup>; kanamycin, 25  $\mu$ g ml<sup>-1</sup> and/or tetracycline, 25  $\mu$ g ml<sup>-1</sup>) were added to the media.

## Results and Discussion

In this manuscript, we have analyzed the compatibility/incompatibility of plasmids containing the same origin of replication in the bacteria *Pseudomonas putida*, and more specifically in *P. putida* U, a strain with a huge catabolic potential [15-19] and with a remarkable biotechnological interest [20-24].

Transformations of *P. putida* U, or its  $\beta$ -oxidation mutant (*P. putida* U  $\Delta$ *fadBA*) with two plasmids containing the same replication origin (pBBR1MCS-2  $Km^R$ ; pBBR1MCS-3  $Tc^R$ ) [6] or with their derivatives carrying  $\beta$ -oxidation genes (*fadA*, *fadB* or both *fadBA*) (Fig. 1) were performed by conjugation using a modification of the mating procedure described by Herrero et al. [11]. After conjugation, colonies showing resistance to the three antibiotics ( $Km$ ,  $Rf$  and  $Tc$ ) were found. Taking into account that  $Rf^R$  is a chromosomal resistance in *PpU*, it could be argued that the other two resistances ( $Km^R$  and  $Tc^R$ ) should be due to either the presence of the two independent plasmids (pBBR1MCS-2 and pBBR1MCS-3) in these bacteria, or to the formation, throughout unusual genetic events, of recombinant plasmids containing two antibiotic resistant genes in a single backbone. Restriction analyses (*Pst*I digestion) of the plasmid(s) isolated from the recombinant *PpU*- $Km^R$ - $Rf^R$ - $Tc^R$  revealed that this

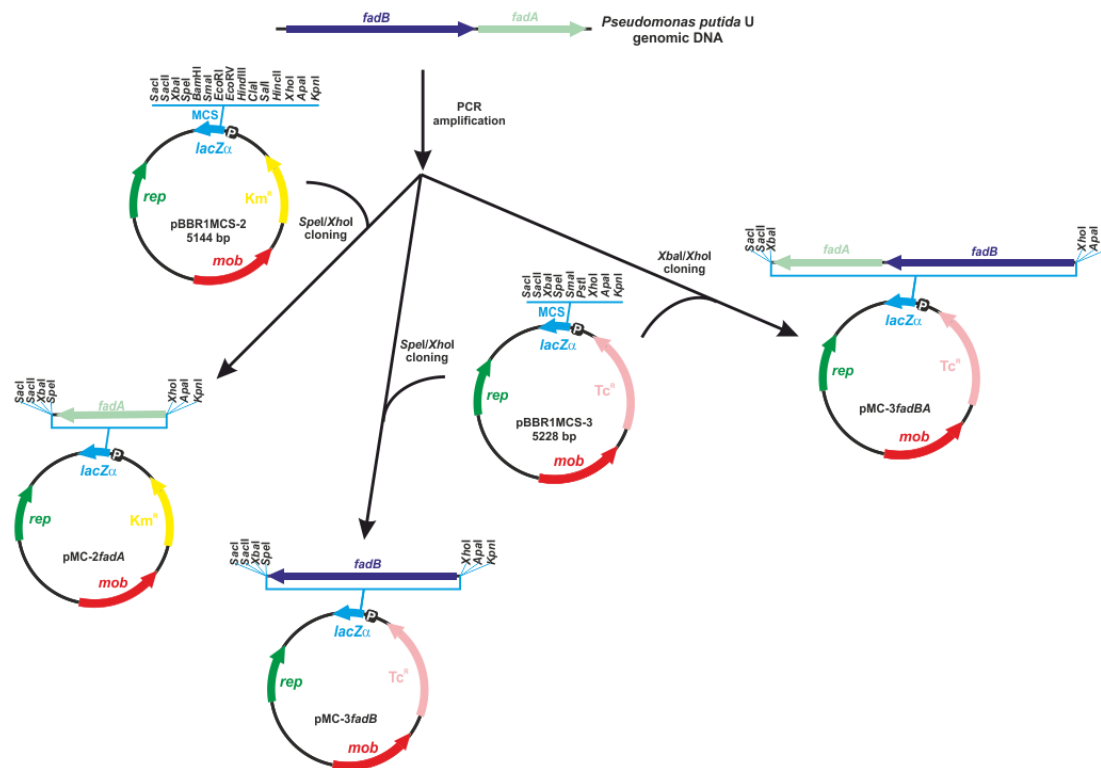
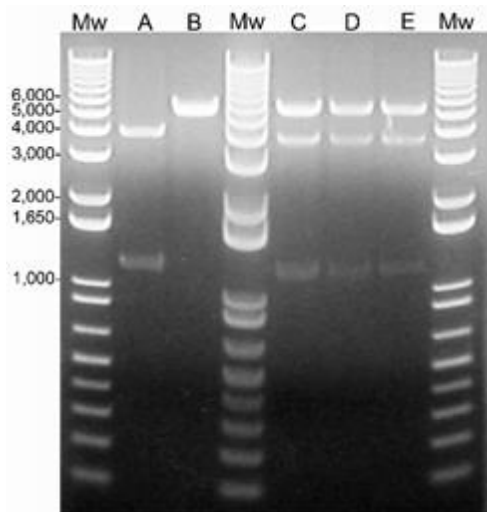


Fig. 1: Maps and construction of the recombinant plasmids derived from pBBR1MCS-2 and pBBR1MCS-3 [6] carrying the genes *fadA*, *fadB*, or *fadBA* from *P. putida* U.

bacterium contains copies of both the plasmids (Fig. 2). Furthermore, when *PpU-Rf<sup>R</sup>-Km<sup>R</sup>-Tc<sup>R</sup>* was re-seeded 40 times (every 24 h) in LB-Km-Rf-Tc, we observed that none of the isolated colonies had lost the resistance to the three antibiotics, showing that, even after cultures, the two plasmids autonomously replicate and co-reside in the same bacterium. Similar results were obtained when *PpU ΔfadBA* and *E. coli* were transformed with the same plasmids (Fig. 2).

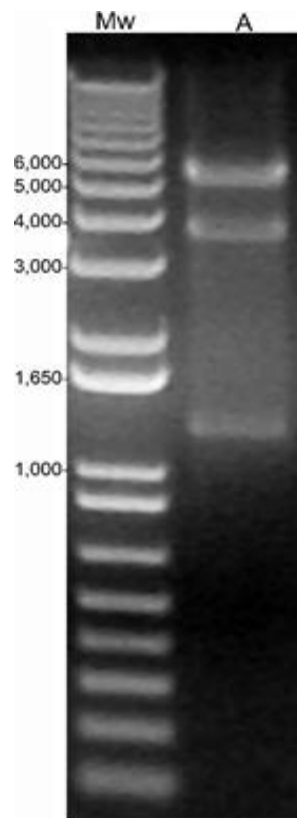


**Fig. 2:** Analysis of the plasmids isolated from recombinant *P. putida* U (*PpU*), *P. putida* U  $\Delta$ *fadBA* (*PpU ΔfadBA*) or from *E. coli* DH10B, when transformed with pBBR1MCS-2 (thereafter pMC-2) (5,144 bp) and pBBR1MCS-3 (from here called pMC-3) (5,228 bp) after being re-seeded in LB medium containing Tc and Km. Electrophoresis were performed in agarose (0.8%) gels embedded in TAE 1X buffer. MW, molecular weight markers (Ready-Load™ 1 Kb Plus DNA Ladder, Invitrogen); A, pMC-2 (Km<sup>R</sup>); B, pMC-3 (Tc<sup>R</sup>); C, plasmids isolated from *PpU* transformed with pMC-2 and pMC-3; D, plasmids isolated from *PpU ΔfadBA* transformed with pMC-2 and pMC-3; E, *E. coli* DH10B transformed with pMC-2 and pMC-3. The DNA fragments generated after *Pst*I digestion of pMC-2 were 3,902 and 1,242; and from pMC-3, 5,228. The numbers on the left indicates the sizes (bp) of some DNA standars.

Conversely, when cells of *PpU-Rf<sup>R</sup>-Km<sup>R</sup>-Tc<sup>R</sup>* were picked-up from single colonies and re-seeded on LB plates containing only one of the antibiotics markers (Km or Tc), we noticed that after six consecutive passes (24 h each one), all the colonies analyzed just kept one plasmid (pBBR1MCS-2 or pBBR1MCS-3, the one that contained the gene of resistance) whereas the other had been excluded. These results showed that, as expected, the persistence of plasmids with the same replication origin in the same bacterium requires an external pressure to be stably inherited [3], and that no recombination events occurred between the plasmids used to transform. When *E. coli* DH10B-Km<sup>R</sup>-Tc<sup>R</sup> was re-seeded in the same conditions indicated above for *PpU-Rf<sup>R</sup>-Km<sup>R</sup>-Tc<sup>R</sup>*, we noticed that all the colonies analyzed maintained the Km and Tc resistances during 10 passes (24 h each one).

These data suggest that despite having the same origin of replication, both plasmids (pBBR1MCS-2 or pBBR1MCS-3) replicate autonomously if the two bacteria (*P. putida* U and *E. coli* DH10B) are subject to selective pressure, whereas in *E. coli* DH10B, unlike

what was believed, plasmids can be stably inherited without any external selection pressure for a longer time (Fig. 3).

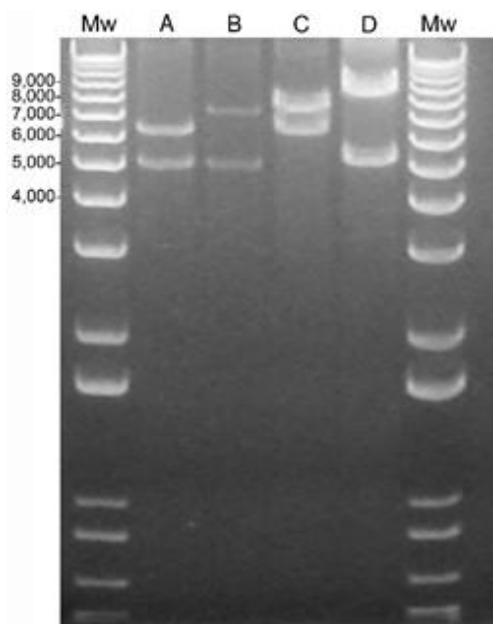


**Fig. 3:** Agarose gel electrophoresis of the plasmids extracted from *E. coli* DH10B pMC-2 pMC-3 after being cultured repeatedly (10 re-seeds) in LB medium containing Km but no Tc (A). MW, molecular weight markers. The same results were obtained when cultured in LB supplemented with Tc but without Km. Plasmids were digested with *Pst*I as indicated in Fig. 2. The numbers on the left indicates the sizes (bp) of some DNA standars.

Although the above results show that the plasmids (pBBR1MCS-2 and pBBR1MCS-3, indicated as pMC-2 and pMC-3) coexisted in *P. putida*, we had not checked whether genetic constructs containing different genes might be efficient and simultaneously expressed in this bacterium. For this purpose, we chose a mutant of *P. putida* (*PpU ΔfadBA*) in which the genes *fadB* and *fadA* encoding the two major  $\beta$ -oxidation proteins (FadB and FadA) have been deleted. This mutant, as already indicated, is unable to grow in chemically defined media [7] having as the sole carbon source n-aryl-alkanoic acids. The transformation of this mutant with different genetic constructions allowed us the collection of the following recombinants: *PpU ΔfadBA* pMC-2*fadA* pMC-3*fadB*, *PpU ΔfadBA* pMC-2*fadA* pMC-3, *PpU ΔfadBA* pMC-2 pMC-3*fadB*, *PpU ΔfadBA* pMC-2 pMC-3, and *PpU ΔfadBA* pMC-2 pMC-3*fadBA* (Figs. 4 and 5).

The incubation of these strains in MM containing 6-phenylhexanoic acid (6-PhH, 10 mM) (hereinafter PhHMM) as the sole carbon source, showed that while strains *PpU ΔfadBA* pMC-2*fadA* pMC-3*fadB* and *PpU ΔfadBA* pMC-2 pMC-3*fadBA* grew well in this

medium, all the others (*PpU ΔfadBA* pMC-2*fadA* pMC-3, *PpU ΔfadBA* pMC-2 pMC-3*fadB* and *PpU ΔfadBA* pMC-2 pMC-3) did not (Fig. 5). These data revealed that the presence of the two β-oxidation proteins FadB and FadA are required for the catabolism of 6-PhH, and that the expression of one of them (FadA or FadB) is not enough to reverse the original phenotype in this mutant. Furthermore, a similar growth was observed when *PpU ΔfadBA* pMC-2 pMC-3*fadBA* and *PpU ΔfadBA* pMC-2*fadA* pMC-3*fadB* were cultured in PhHMM, suggesting that the expression of FadB and FadA from a single plasmid construction (pMC-3*fadBA*) or from two plasmids (pMC-2*fadA* pMC-3*fadB* that replicates autonomously in *PpU ΔfadBA*) was enough to assure the degradation of 6-PhH (Fig. 5).

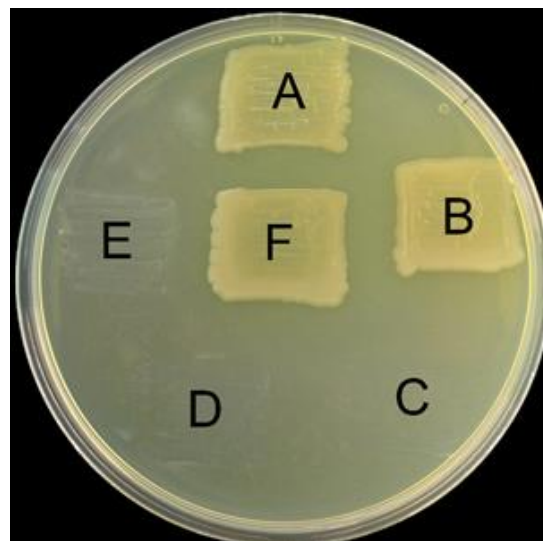


**Fig. 4:** Agarose gel electrophoresis of the plasmids extracted from A, *PpU ΔfadBA* pMC-2*fadA* pMC-3; B, *PpU ΔfadBA* pMC-2 pMC-3*fadB*; C, *PpU ΔfadBA* pMC-2 *fadA* pMC-3*fadB*; D, *PpU ΔfadBA* pMC-2 pMC-3*fadBA*. Bacteria were re-seeded in LB supplemented with Tc and Km. Digestions of the different plasmid with *XhoI*, generate the following DNA fragments: pMC-2, 5,144; pMC-3, 5,228; pMC-2*fadA*, 6,446; pMC-3*fadB*, 7,757; pMC-3*fadBA*, 8,678 bp. The numbers on the left indicates the sizes (bp) of some DNA standars.

The characterization of the plasmids in these recombinants showed that all they contained the same constructions used to transform *PpU ΔfadBA* (Fig. 4). Additionally, we observed that the transformation of *PpU ΔfadBA* with two constructs of the same plasmid (pMC-3) carrying each of them a single β-oxidation gene (pMC-3*fadB* or pMC-3*fadA*), restores in the recombinant bacteria (*PpU ΔfadBA* pMC-3*fadB* pMC-3*fadA*), the ability to degrade 6-phenylhexanoic acid (Fig. 5). These results show that constructs derived from a single plasmid, even wearing the same antibiotic resistance, can be used effectively in those experiments in which the function of more than one gene should be restored.

When *E. coli* was transformed with the plasmids (or plasmid derivatives) collected from different *PpU* recombinants, the same

results were obtained suggesting that the genetic constructions derived from a single plasmid are also compatible in this bacterium.



**Fig. 5:** Bacterial growth of *PpU ΔfadBA* pMC-2*fadA* pMC-3*fadB* (A); *PpU ΔfadBA* pMC-2 pMC-3*fadBA* (B); *PpU ΔfadBA* pMC-2*fadA* pMC-3 (C); *PpU ΔfadBA* pMC-2 pMC-3*fadB* (D); *PpU ΔfadBA* pMC-2 pMC-3 (E); and *PpU ΔfadBA* pMC-3*fadA* pMC-3*fadB* (F) when cultured in a chemically defined medium containing 6-phenylhexanoic acid (10 mM) as the sole carbon source.

## Conclusion

The use of plasmids with the same origin of replication as genetic vectors has certain advantages over the traditional methods that employ unrelated plasmids to express simultaneous, and independently, more than one protein in the same bacterium. This experimental approach is especially useful when complementation studies are addressed (see the results showed above). Thus, in those cases in which two or more proteins have been affected by mutations, the transformation of these cells with a family of independent constructs (built in the same plasmid) assures: (i) a similar number of plasmid copies, and (ii) close expression rates of the cloned genes (all them are under the control of the same promoter).

Furthermore, these data also have certain ecological interest since the use of plasmids derivatives containing the same backbone, but different inserts, reduces the risk of release to the environment those genes encoding resistance to different antibiotics or to other chemicals.

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## Conflict of Interest

There is no conflict of interest with either institutions or individuals.

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