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**Master in Molecular Biology and Biochemistry**

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**Mobilization of the ColE1 Plasmid  
with Synthetic Constructs**

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# Introduction

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The horizontal gene transfer (HGT) is the movement of genetic material between unicellular and/or multicellular organisms other than the vertical transmission (the transmission of DNA from parent to offspring).

Widespread horizontal gene transfer has profound evolutionary implications, as it allows homologous recombination between closely species in a process resembling eukaryotic sex (Frost *et al*, 2005). Also, it leads to the integration of new genetic information, creating large functional leaps that allow fast adaptation to new environments or to stressful conditions (Guasch *et al*, 2003). Furthermore, gene mobility has been proposed to drive microbial cooperative processes (Nogueira *et al*, 2009). There are three known mechanisms of horizontal transfer: transformation, transduction, and bacterial conjugation, the latter is thought to be the most important (Halary *et al*, 2010) and is a specialized gene transfer process that involves unidirectional transfer of DNA from donor to recipient bacteria by a mechanism that requires cell-to-cell contact.

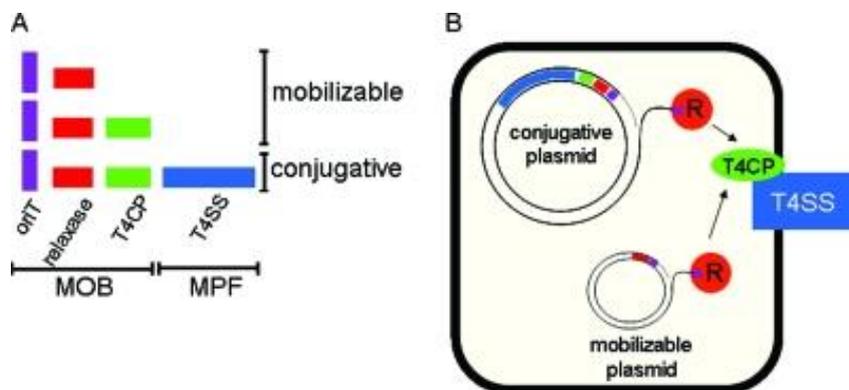
Plasmids, and integrative conjugative elements (ICEs), are the main elements in bacterial conjugation processes. They spread both within and between bacterial species carrying with them various genes, allowing bacteria to acquire new abilities. Some of these abilities, just to name few, include resistance to toxic organic compounds, such as antibiotics, are carried by plasmids (Barlow, 2009). They also code for information essential for the interaction of bacteria with multicellular eukaryotes, including nitrogen fixation by rhizobia (Long, 1989), plant cell manipulation by *Agrobacterium* species (Gelvin, 2009), and virulence among many human pathogens.

The horizontal spread of plasmids may occur by bacterial conjugation if a plasmid carries two specific sets of genes.

1. The set of mobility (MOB) genes is essential and allows conjugative DNA processing. They usually are a relaxase gene, and one or more nicking auxiliary proteins.
2. A set of genes that code for a membrane-associated mating pair formation (MPF) complex, which usually it is composed by the type IV coupling protein (T4CP) and the components of the mating channel that assemble a T4SS. The T4CP is involved in the connection between the relaxosome and the transport channel (Mihajlovic *et al*, 2010). The conjugative

mating channel is basically a protein secretion channel, which transports the relaxase protein bound to the DNA to be transferred (Draper *et al*, 2005). According to the nomenclature of protein secretion mechanisms, it is a T4SS (Christie *et al*, 2005).

A plasmid that codes for its own set of MPF genes is called self-transmissible or conjugative. If it uses an MPF of another genetic element present in the cell, it is called mobilizable. Some plasmids are nonmobilizable because they are neither conjugative nor mobilizable and they spread by natural transformation or by transduction. Hence, plasmids can be classified into three categories according to mobility: conjugative, mobilizable, and nonmobilizable. Conjugative plasmids tend to be large (>30 kb) with low copy number, while mobilizable plasmids are small (<15 kb) and have high copy number.



**Figure 1.** Overview of plasmid mobilization components. **(A)** Schematic view of the genetic constitution of transmissible plasmids. Self-transmissible or conjugative plasmids code for the four components of a conjugative apparatus: an origin of transfer (*oriT*) (violet), a relaxase (R) (red), a type IV coupling protein (T4CP) (green), and a type IV secretion system (T4SS) (blue). Mobilizable plasmids contain just a MOB module (with or without the T4CP) and need the MPF of a cohabitant conjugative plasmid to become transmissible by conjugation. **(B)** Scheme of some essential interactions in the process of conjugation. The relaxase cleaves a specific site within *oriT*, and this step starts conjugation. The DNA strand that contains the relaxase protein covalently bound to its 5' end is displaced by an ongoing conjugative DNA replication process. The relaxase interacts with the T4CP and then with other components of the T4SS. As a result, it is transported to the recipient cell, with the DNA threaded to it. Subsequently, the DNA is pumped into the recipient by the ATPase activity of the T4CP. Figure adapted from Smillie *et al*, 2010.

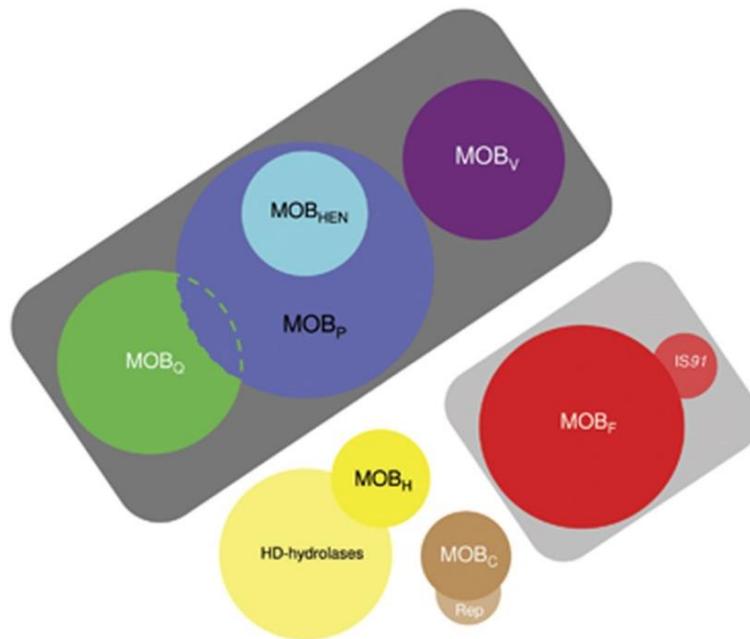
The only protein component of the conjugative machinery that is common to all transmissible, i.e., conjugative or mobilizable, plasmids is the relaxase (Figure 1). The relaxase is a key protein in conjugation. It recognizes the origin of transfer (*oriT*), a short DNA sequence which is the only sequence required in *cis* for a plasmid to be conjugally transmissible. The relaxase catalyzes the initial and final stages in conjugation, that is, the initial cleavage of *oriT* in the donor, to ultimately produce the DNA strand that will be transferred, and the final ligation of the transported DNA in the recipient cell that reconstitutes the conjugated plasmid (de la Cruz, 2010). Conjugative relaxases are structurally related to rolling-circle replication initiator proteins, and they catalyze similar biochemical reactions.

## **MOB families**

As mentioned above, generally all transmissible plasmids contain a MOB region, required for mobilization, while self-transmissible plasmids contain, on top of that, a T4SS that allows the assembly and functionality of the mating channel. Conjugative plasmids have been classified in six MOB families according to their transfer systems: MOB<sub>F</sub>, MOB<sub>H</sub>, MOB<sub>Q</sub>, MOB<sub>C</sub>, MOB<sub>P</sub> and MOB<sub>V</sub> (Figure 2).

Relaxase proteins are large and usually contain two or more protein domains. The relaxase domain proper is located always at the N-terminus of the protein. At the C-terminus, a DNA helicase, DNA primase or other domain of unknown function is almost always found. Most relaxases show a significant degree of sequence similarity as well as three conserved amino acid sequence motifs. Motif I contains one or two tyrosines, which are considered the catalytic sites of the relaxase. Motif II contains a serine implicated in the interaction of the relaxase with the 3'-end of the *nic* DN. Motif III contains a histidine triad that the protein uses to bind divalent cations (called the 3H-motif) and has been used as a relaxase diagnostic signature.

In some cases, relaxases contain a signature variant composed by a histidine, a glutamate and an asparagine (called the HEN-motif) (Francia et al, 2004 and references herein). Due to their sequence similarities, relaxases can be used as phylogenetic tools for plasmid classification.



**Figure 2.** A scheme of the relationships between the main relaxase protein families. A first relaxase cluster (shown on a dark-grey background) contains relaxase groups that contain just one active Tyr in the catalytic center. A second relaxase cluster (light-grey) contains relaxases with two Tyr in the catalytic center. For the remaining groups (white background) not enough is known about the biochemistry of the respective relaxases. Some relaxase protein families also overlap other protein families, such as plasmid RC-replication proteins (Rep). Areas of circles are proportional to relaxase number. The MOB<sub>P</sub> area includes (MOB<sub>P</sub>+MOB<sub>HEN</sub>+MOB<sub>Q1</sub>). Adapted from Garcillán-Barcia et al, 2009.

## Plasmid incompatibility

Plasmid incompatibility is the inability of two different plasmids to coexist stably in the same host cell in the absence of continued selection pressure. In simpler terms, if the introduction of a second plasmid negatively affects the inheritance of the first, the two are considered to be incompatible. One may speak of incompatibility only when it is certain that entry of the second plasmid has taken place, and where DNA restriction is not involved. Groups of plasmids that are mutually incompatible with one another have been variously referred to as incompatibility groups.

Incompatibility may be symmetric (either co-resident plasmid is lost with equal probability) or vectorial (one plasmid is lost exclusively or with higher probability than the other). Although certain plasmid elements can cause either type, it is suggested that the statistical mechanisms are

slightly different. Symmetric incompatibility is seen with co-resident single replicons that share essential replication and maintenance functions and is due to inability to correct fluctuations arising as a consequence of the random selection of individual copies for replication and partitioning events within the plasmid pool.

Vectorial incompatibility is usually due to interference with replication by cloned plasmid fragments containing elements of the replication control or maintenance systems or by certain copy control mutations of directly regulated plasmids. Sometimes the replication of the affected plasmid is completely blocked; more often the block is partial, or even minimal, and it is proposed that in such cases loss of the plasmid is due to inability to correct fluctuations arising as a consequence of the random temporal distribution of replication events. It is noted that, with unit copy plasmids such as F and P1, it is impossible to analyze co-resident incompatible plasmids simply because the copy numbers are too low to permit the construction of heteroplasmid strains.

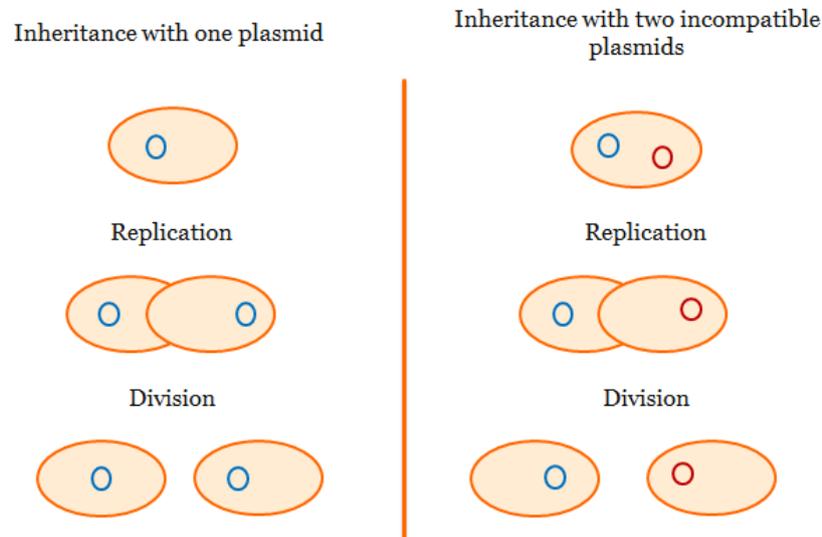
It is widely accepted that competition for replication factors leads to competition between plasmids. Plasmids with inherent growth advantages such as faster replication (due to their smaller size) or less toxicity have the potential to rapidly outgrow other plasmids in the cell. This is even more likely to occur when the plasmid copy number is low.

Plasmids vary widely in their copy number (see Table 1) depending on the origin of replication they contain, which determines whether they are under relaxed or stringent control; as well as the size of the plasmid and its associated insert. Some plasmids, such as the pUC series and derivatives, have mutations which allow them to reach very high copy numbers within the bacterial cell, whereas plasmids based on pBR322 and many cosmids are generally maintained at lower copy numbers. Very large plasmids are often maintained at very low copy numbers per cell. In this study, series of pUC derived vectors were used.

<b>DNA construct</b>	<b>Origin of replication</b>	<b>Copy number</b>	<b>Classification</b>
pUC vectors	pMB1*	500–700	High copy
pBluescript vectors	ColE1	300–500	High copy
pGEM vectors	pMB1*	300–400	High copy
pTZ vectors	pMB1*	>1000	High copy
pBR322 and derivatives	pMB1*	15–20	Low copy
pACYC and derivatives	p15A	10–12	Low copy
pSC101 and derivatives	pSC101	~5	Very low copy

**Table 1.** Origin of replication and copy number of several plasmids. Adapted from Quiagen©.

Taking into account the number of cell division cycles occurring in a single overnight culture, even small differences in competitive advantage are thought to be able to lead to rapid dominance of a culture by a single plasmid.



**Figure 3.** Comparison between the inheritance of a single plasmid, or compatible plasmids, and inheritance of two incompatible plasmids. Copy number and plasmid incompatibility are linked. Incompatible plasmids cannot be distinguished from each other at the point of replication initiation, and are therefore distributed to different daughter cells.

### **ColE1 and its MOB region**

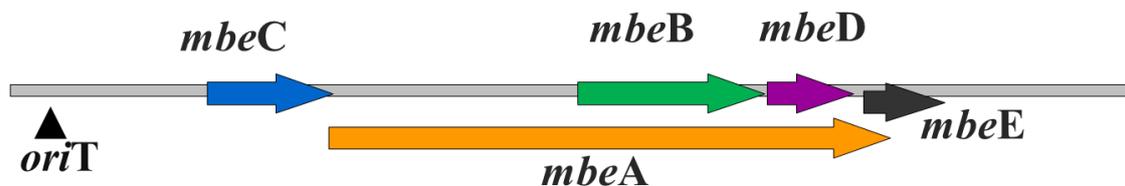
ColE1 is one of the best known plasmids because of its extensive use in the construction of cloning and expression vectors. It is mobilized by a wide array of conjugal plasmids. This ability attracted considerable scientific attention and reports on its conjugal mobilization were first published in the late '60s (Clewell and Helinski, 1969).

However, studies on the function and interactions of the mobilization proteins involved were abandoned for several years and the original location of the cis-acting *oriT* and *nic* sites of ColE1 were under dispute (Bastia, 1978). At the end of the 20th century, the return of persistent bacterial infections due to the development of multi-drug resistance and its connection with

horizontal gene transfer, re-attracted scientific interest in deeper studies on conjugal mobilization, thereby re-igniting interest in ColE1.

Its mobilization region (Figure 4) consists of a cluster of five genes (*mbeA*, *mbeB*, *mbeC*, *mbeD* and *mbeE*) coding for the following proteins, respectively:

- ❖ **MbeA**, the relaxase of ColE1, binds specifically to *oriT* and splits the phosphodiester bond at a site designated *nic* (Varsaki *et al*, 2003). It's the prototype of the HEN-type relaxases.
- ❖ **MbeB** has been proposed to be involved in relaxosome functions (Lovet and Helinski, 1975). Up to date, its exact function is unknown.
- ❖ **MbeC** is a dimer and probably belongs to a ribbon-helix-helix family of nicking accessory proteins (Varsaki *et al*, 2009). Its role is dual, as *mbeC* binds, through its N-terminal region, specifically on the *oriT* dsDNA and through its C-terminal region binds with the N-terminal region of *mbeA* (Varsaki *et al*, 2012).
- ❖ **MbeD** has been proposed as an “entry exclusion” protein (Yamada *et al*, 1995).
- ❖ **MbeE** characterized as non-essential for the ColE1 plasmid mobilization (Boyd *et al*, 1989).

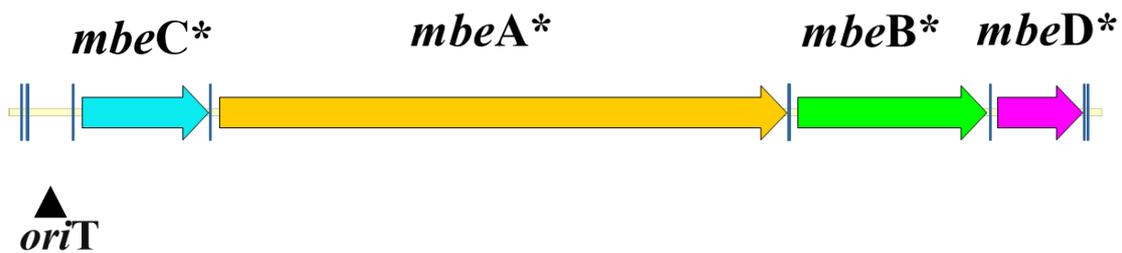


**Figure 4.** Genetic structure of the ColE1 mobilization region. It is composed of the five *mbe* genes.

The organization of the mobilization region of ColE1 plasmid is complex and quite unusual, as it is not common for plasmids to have overlapping genes. Since *mbeB* and *mbeD* completely overlap *mbeA* (Boyd *et al*, 1989) in a complex structure, genetic manipulation of *mbeB* or *mbeD* gene in order to elucidate their role in mobilization of ColE1 plasmid, without affecting *mbeA* is complicated.

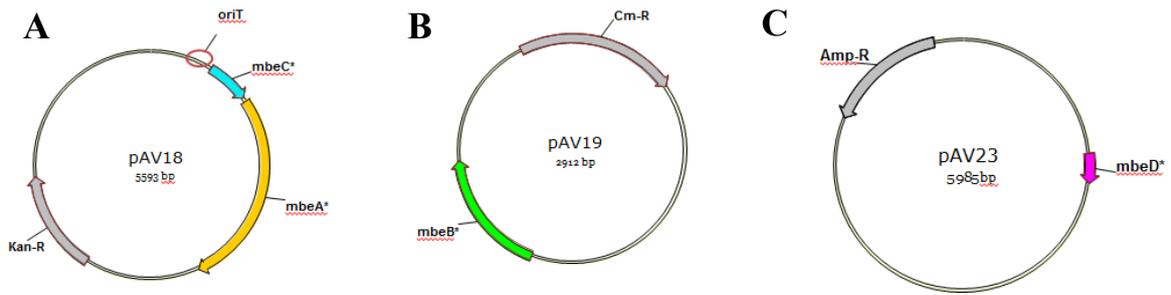
For that reason, the mobilization region of ColE1 plasmid has been synthetically reconstructed (Varsaki A., unpublished data) as four gene fragments (gBlocks from Integrated DNA Technologies) which were assembled into one construct using the Gibson Assembly Method (Gibson *et al*, 2009). For the design of the gBlocks, the ELP 2.0 software was used, which allows the design of synonymous sequences with maximally divergent evolutionary potentials relative to the input sequences (Cambray and Mazel, 2008).

The construct comprised four synthetic genes encoding proteins identical to the ones coded by *mbeA*, *mbeB*, *mbeC* and *mbeD*. Contrary to the wild type organization, synthetic *mbeB* (*mbeB\**) and synthetic *mbeD* (*mbeD\**) were not overlapping the synthetic *mbeA* (*mbeA\**), but were located downstream, as shown in Figure 5. Unique restriction sites, upstream and downstream each gene, allow easy manipulation.



**Figure 5.** Synthetic construct of the ColE1 mobilization region (ColE1-MOB<sup>S</sup>). Contains the *oriT* at the beginning and the synthetic genes *mbeC\**, *mbeA\**, *mbeB\** and *mbeD\** in parallel. Blue bars indicate unique restriction sites.

Surprisingly, it proved impossible to clone the construct shown in Figure 5 (ColE1-MOB<sup>S</sup>) using the plasmid pSU18 as cloning vector. Nevertheless, ColE1-MOB<sup>S</sup> was successfully cloned as functional independent fragments, resulting in plasmids pAV18 (*oriT-mbeC\*-mbeA\**), pAV19 (*mbeB\**) and pAV23 (*mbeD\**) (Figure 6 A, B and C, respectively) (Varsaki A., unpublished data). The fragment (*mbeB\* + mbeD\**) proved impossible to clone, using plasmid pSU18 as cloning vector. All the clones containing the synthetic genes were coding for functional proteins MbeA\*, MbeB\*, MbeC\* and MbeD\* (Varsaki A., unpublished data).



**Figure 6.** Map of the plasmids holding the synthetic constructions. **(A)** pAV18 (5593 bp) has a kanamycin resistance, the *oriT* and the synthetic genes *mbeC\** and *mbeA\**. **(B)** pAV19 (2912 bp) has a chloramphenicol resistance and the synthetic gene *mbeB\**. **(C)** pAV23 (5985 bp) has a chloramphenicol resistance and the synthetic gene *mbeD\**.

The inability to clone the whole *ColE1-MOB<sup>S</sup>* region as well as the *mbeB\** + *mbeD\** in a single plasmid raised several questions about those two genes. In order to gather more information we decided to focus in their wild type version, *mbeB* and *mbeD*, and see their effect when cohabiting with the synthetic counterpart of the *MOB* region.

# Objectives

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- ❖ Built a plasmid containing the wild-type (*mbeB+mbeD*) compatible with pAV18 (*oriT-mbeC<sup>\*</sup>-mbeA<sup>\*</sup>*).
- ❖ Test if it complements pAV18 (*oriT-mbeC<sup>\*</sup>-mbeA<sup>\*</sup>*).
- ❖ Compare the conjugation frequencies with wild-type strains and strains lacking the *mbeB* and *mbeD* genes.

# Methodology

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## **Bacterial strains, plasmids and bacterial growth conditions**

Plasmids used are listed in Table 2. *Escherichia coli* strains used were DH5 $\alpha$  (F<sup>-</sup> *endA1 hsdR17 supE44 thi-1 recA1  $\Delta$  (argF-lacZYA) u160  $\phi$ 80d lacZ  $\Delta$  M15 gyrA96*), and HMS174 (*recA1 hsdR rif<sup>r</sup>*). Bacteria were grown at 37 °C in LB broth (Sambrook *et al.*, 1989), supplemented with 2% (w/v) agar for growth on plates. When indicated, growth media were supplemented with antibiotics at the following concentrations: sodium ampicillin (Ap; 100  $\mu$ g/ml), chloramphenicol (Cm; 25  $\mu$ g/ml), nalidixic acid (Nx; 20  $\mu$ g/ml), rifampicin (Rif; 50  $\mu$ g/ml), tetracycline (Tc; 10  $\mu$ g/ml) and trimethoprim (Tp; 10  $\mu$ g/ml), kanamycin sulfate (Km) 50  $\mu$ g/ml.

Plasmid	Antibiotic Selection	Description	Size (bp)	MOB	Inc group/Ori gin of replication	Source, Reference or Construction Strategy
pSU18	Chloramphenicol	Cloning Vector	2,300	-	P5	Bartolomé <i>et al</i> (1991)
pAV18	Kanamycin	pCR-blunt::(oriT+mbeA*+mbeC*)	5,593	ColE1	PUC	Varsaki, A. Unpublished data
pSU4601	Kanamycin	ColE1::Km	7,930	P5	ColE1	Cabezón <i>et al</i> , (1997) Varsaki, A.
pAV19	Chloramphenicol	pSU18::mbeB*	2,912	-	P15A	Unpublished data
pAV23	Ampicillin	pRRG8::mbeD*	5,985	-	RK2	Varsaki, A. Unpublished data
pMRC1	Chloramphenicol	pSU18::mbeB+mbeD	3,144	ColE1	P15A	This study
R388	Trimethoprim	Devoid of transposable elements	33,926	F11	W	Datta, N. & Hedges, R. W. (1972)
pRL443	Ampicillin	Derivative of RP4	52,800	P11	P1α	Elhai, J <i>et al</i> , (1997)
R64-drd11	Tetracycline	R64 Derepressed for transfer	56,700	P12	I1α	Komano <i>et al</i> , (1990)
R751	Trimethoprim	Derivative of RP4	56,000	P11	P1β	Jobanputra RS & Datta N. (1974)
R6K-drd1	Ampicillin	Derepressed mutant of R6K	28,500	P3	X2	Avila <i>et al</i> ,(1996)

**Table 2.** Conjugative and mobilizable plasmid used in this study.

## Plasmid methodology, enzymes and oligonucleotides.

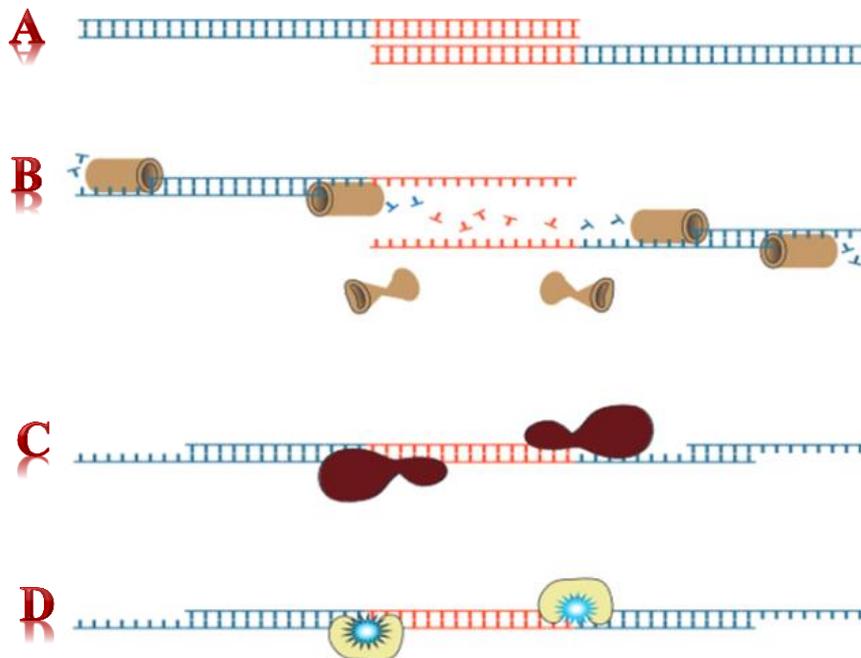
Plasmid DNA was purified in a small scale using the GeneJET Plasmid Miniprep Kit from Thermo Fisher Scientific (Catalog code: K0503). DNA fragments were purified from agarose gels using the GeneJet Extraction Kit from Thermo Fisher Scientific (Catalog code: K0692). Nanodrop measures were performed with a Nanodrop®2000c from Thermo Fisher Scientific. For PCR-amplification the Phusion Polymerase from Thermo Fisher Scientific (Catalog code: F530S/L) was used. Cloning techniques were carried out by standard methodology (Sambrook et al., 1989). T4 DNA ligase was bought from New England Biolabs. Restriction endonucleases (FastDigest) were purchased from Thermo Fisher Scientific. Oligonucleotides used are listed on Table 3.

Oligonucleotide Name	Sequence
<b>pAV21-plus-nuevo</b>	5' GATCCTCTAGAGTCGACCTGCAG 3'
<b>pAV21-minus</b>	5' GTGTACATTCGACTCACCGTGGAC 3'
<b>BD-plus</b>	5' CTGAAAGGAGCATCATATGAGCA 3'
<b>BD-minus-nuevo</b>	5' CTGAAAGGAGCATCATATGAGCA 3'
<b>Ref-comp-plus 1</b>	5' CACACAGGAAACAGCTATGACCA 3'
<b>Ref-comp-minus 2</b>	5' CAGTCACGACGTTGTAAAACGAC 3'

**Table 3.** List of oligonucleotides used

## Gibson assembly

The Gibson Assembly Method (Gibson et al, 2009) (Figure 7) is a fast assembly method that provides directional cloning of multiple DNA fragments in a single reaction conducted during 1h at 50°C, without the need for specific restriction sequences. It relies on use of an enzyme mixture consisting of an exonuclease, a ligase, and a high-fidelity polymerase. For the assembly reaction, the gBlocks Gene Fragments and the vector insertion site are designed with overlapping sequences at the locations that are to be joined. At 50°C, the exonuclease digests dsDNA from the 5' ends, but is rapidly degraded leaving complementary, 3' ssDNA ends. The resulting single-stranded, complementary ends are then available to hybridize to each other, at which point the polymerase fills in missing nucleotides and the ligase covalently joins the fragments together.



**Figure 7.** Gibson Assembly reaction with 2 gBlocks® Gene Fragments and a generic plasmid. (A) dsDNA fragments (gBlocks) with overlapping ends. (B) Exonucleases (in brown) digest the 5' DNA ends, and are rapidly inactivated by the 50°C temperature. (C) Complementary overhangs anneal, and high-fidelity polymerases (in red) fill in any gaps. (E) In the final step, a ligase covalently joins DNA fragments. Figure adapted from Integrated DNA Technologies®.

## **Conjugation experiments**

Cultures were grown overnight at 37 °C in LB with appropriate antibiotics for plasmid selection. Then were diluted 1:100 and left to grow until  $A_{600}=0.4-0.6$ . *E. coli* strain DH5 $\alpha$  carrying the appropriate plasmids was used as donor and HMS174 as recipient. Donor and recipient cells were washed from antibiotics, mixed 1:1 and placed on a 25-well microtiter plate, filled with 1ml LB agar in each well. The microtiter plate was placed at 37 °C for 2 h. Then bacteria were washed from the agar surface with LB broth, diluted with LB broth and plated on selective media. Transfer frequencies are expressed as the number of transconjugants per recipient cell.

## **Standard genetic experiments**

Bacterial transformation was carried by electroporation (Dower *et al.*, 1988). Highly efficient competent cells were prepared (Hanahan, 1983).

# Results

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## Construction of the pMCR1 plasmid

Plasmid pSU18 was selected as vector and the wild-type genes *mbeB* and *mbeD* as insert. Cloning was performed by Gibson Isothermal Assembly and following standard cloning procedures, as described at the section of “Methodology”.

Two PCRs were performed, one for insert and one for vector amplification. PCR mixture preparation followed standard procedures. Primers for the amplification of pSU18 were: pAV21-plus-nuevo and pAV21-minus. For the amplification of *mbeB* + *mbeD* from ColE1, the plasmid pSU4601 was used as template and the primers BD-plus and BD-minus-nuevo. The PCR program performed was:

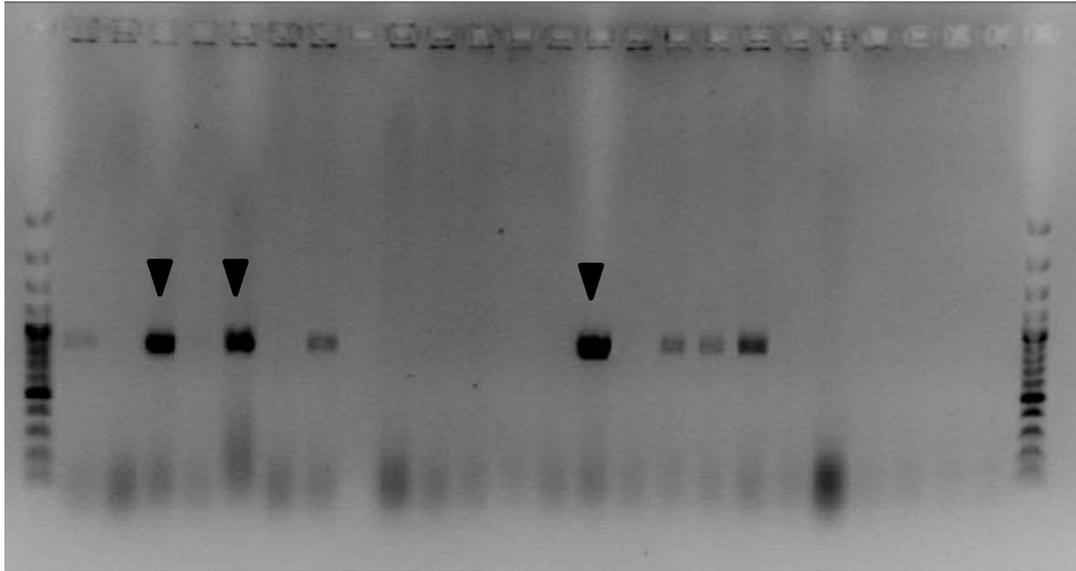
95 C°/ 5 min; [(95 C°/ 45 sec; 65 C°/ 1 min;72 C°/ 1 min)x30]; 72 C°/ 10 min

An electrophoresis was conducted for 45 min at 120V, expecting bands of 900bp for the insert and 2,400bp for the vector. The DNA was extracted from the gel and cleaned. The concentration was measured with a Nanodrop®2000c from Thermo Fisher Scientific.

Digestion with the enzyme DpnI (FastDigest DpnI from Thermo Fisher Scientific, catalog code FD1703) was performed on the pSU18 plasmid according to the instructions provided in the catalog. The reaction was set for 2h at 37°C with an inactivation phase of 5min at 80°C. Sample was then cleaned with the GeneJET Plasmid Miniprep Kit from Thermo Fisher Scientific (Catalog code: K0503). Concentration measures were taken again.

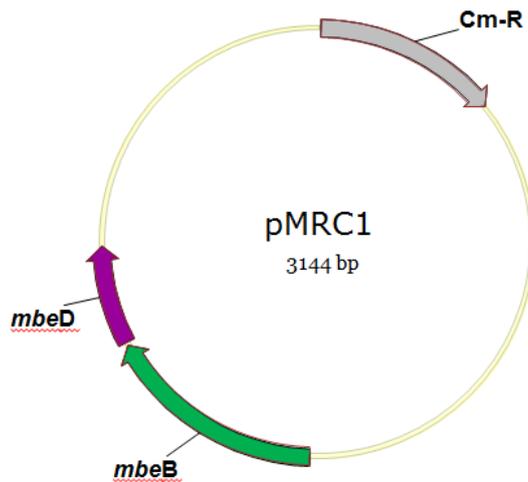
Isothermal (also known as Gibson Assembly) was then performed according to the protocol as stated above (Figure 7). A 1:1 insert:vector ratio was added to the isothermal mix aliquots prepared in the lab to a final volume of 20µl. then left in a thermo cycler for 1h at 50°C. A negative control was also used containing only the vector and then filled to 20µl with distilled water.

Primers used for the isothermal experiments were: Ref-comp plus 1 and Ref-comp-minus 2. Samples were then electroporated into *E. coli* strain DH5α. Bacteria were left in a LB agar petri dish (20ml LB agar + 1µl/ml chloramphenicol) grown overnight. Next, a verification colony PCR showed three colonies potentially contained the construct, so a small sample of each was sent for sequencing (Figure 8).



**Figure 8.** Result of the colony PCR. Eight colonies showed the desired band at approximately 3,000bp in the molecular ladder. Samples three, five and fourteen (black arrows) were sent for sequencing.

Sequencing results were blasted against the wild-type ColE1 with satisfactory results. This new plasmid was named pMRC1 (Figure 9).



**Figure 9.** Map of the pMRC1 plasmid. It contains a chloramphenicol resistance and the wild-type *mbeD*+*mbeB* genes. Length is 3,144 bp.

## Conjugation experiments

They were performed in order to test the degree of complementation between pAV18 and pMRC1. Conjugative plasmids used were R388, pRL443, R6K-drd1, R64-drd11 and R751 (Table 2). Donors were strain DH5 $\alpha$  carrying the appropriate plasmids and strain HMS174 was used as receptor.

Cultures were grown overnight without shaking at 37°C in LB with appropriate antibiotics for plasmid selection until A600 = 0.6 Escherichia coli strain DH5 $\alpha$  carrying the appropriate plasmids were used as donor and HMS174 as recipient. DH5 $\alpha$  (0.3 ml) carrying the appropriate plasmids and 0.3 ml HMS174 were centrifuged separately at 4000 r.p.m. for 10 min and washed from the antibiotics with LB medium. Then cells were mixed and placed onto different well of a 24-wells plate with 1ml of solidified LB agar, depending on their conjugative plasmid, for 2 h at 37°C. Then they were resuspended with 1ml PBS and made into serial dilutions (1, 1/10–1/100000000) and transferred to LB agar petri dishes with appropriate antibiotic(s) for their selection. Selection of donors, receptors and transconjugants was designed as follows:

DH5 $\alpha$ /R388 + pAV18 + pMRC1 x HMS174	}	Donors (Chloramphenicol + Kanamycin)
	}	Receptors (Rifampicin)
	}	Transconjugants (Rifampicin + Kanamycin)

DH5 $\alpha$ /pRL443 + pAV18 + pMRC1 x HMS174	}	Donors (Chloramphenicol + Kanamycin)
	}	Receptors (Rifampicin)
	}	Transconjugants (Rifampicin + Kanamycin)

D1210 /R64-drd1 1 + pAV18 + pMRC1 x HMS174	}	Donors (Chloramphenicol + Kanamycin)
	}	Receptors (Rifampicin)
	}	Transconjugants (Rifampicin + Kanamycin)

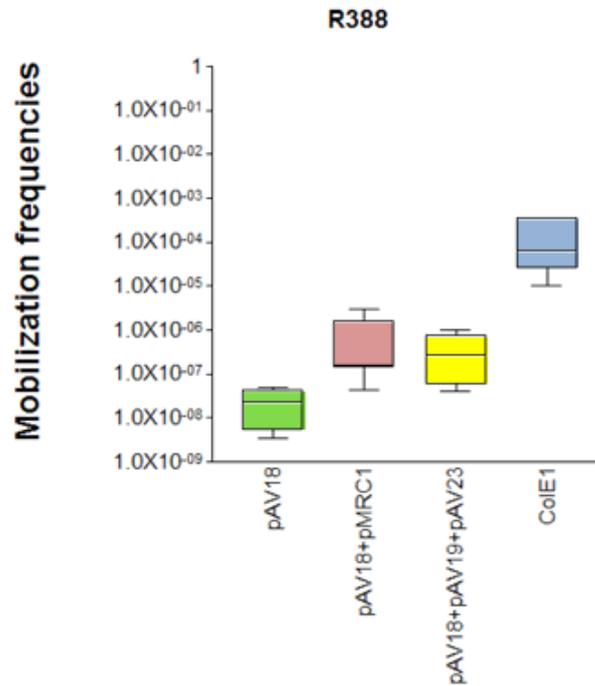
DH5 $\alpha$ /R6K-drd1 1 + pAV18 + pMRC1 x HMS174	}	Donors (Nalidixic acid + Kanamycin)
	}	Receptors (Rifampicin)
	}	Transconjugants (Rifampicin + Kanamycin)

DH5 $\alpha$  /R751 + pAV18 + pMRC1 x HMS174 } Donors (Nalidixic acid + Kanamycin)  
 } Receptors (Rifampicin)  
 } Transconjugants (Rifampicin + Kanamycin)

Conjugation Frequency (CF) calculation for donors or receptors (DoR): Colony Forming Units (CFU) in the dishes are counted , then a ratio is performed in order to obtain a conjugation frequency, the higher this ratio is, the better these mobilizable plasmids are at reaching receptor cells.

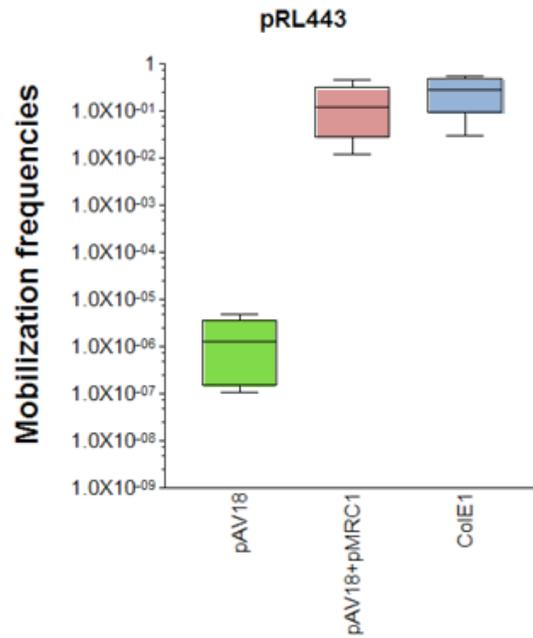
$$CF = \frac{T}{D \text{ or } R}$$

The mobilization frequencies of the synthetic constructs and the ColE1 plasmid were calculated by conjugation assays, using various conjugative plasmids. Values are shown in Figures 10 to 14. Results are the average of at least 6 repetitions. Experiments with the plasmids [pAV19 (*mbeB*<sup>\*</sup>) + pAV23 (*mbeD*<sup>\*</sup>)] were discontinued due lack of time.



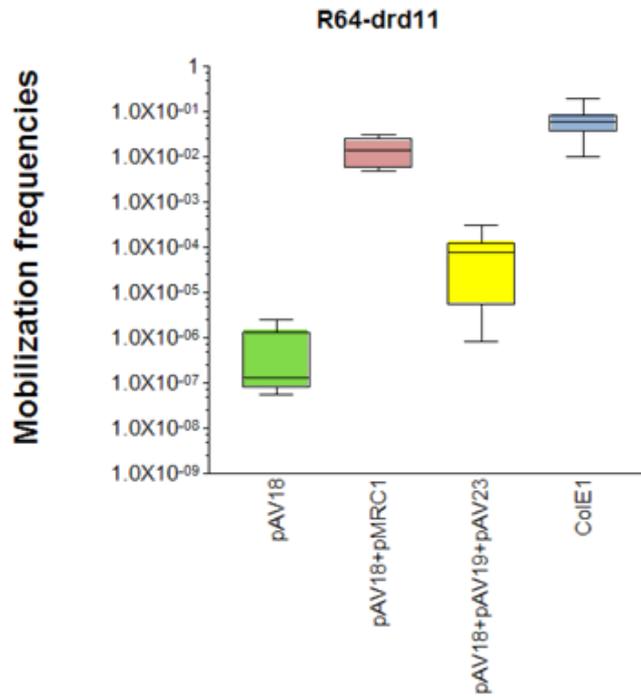
**Figure 10.** Mobilization frequencies using the R388 conjugative plasmid. Green: pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*]. Red: pMRC1 [wt-*mbeC* + wt-*mbeD*] + pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*]. Yellow: pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*] + pAV19 (*mbeB<sup>S</sup>*) + pAV23 (*mbeD<sup>S</sup>*). Blue: wild-type ColE1 plasmid.

pAV18 is in the -7 order of magnitude, pAV18 + pMRC1 in the -6, pAV18 + pAV19 + pAV23 in the -6 and the wild type ColE1 in the -4.



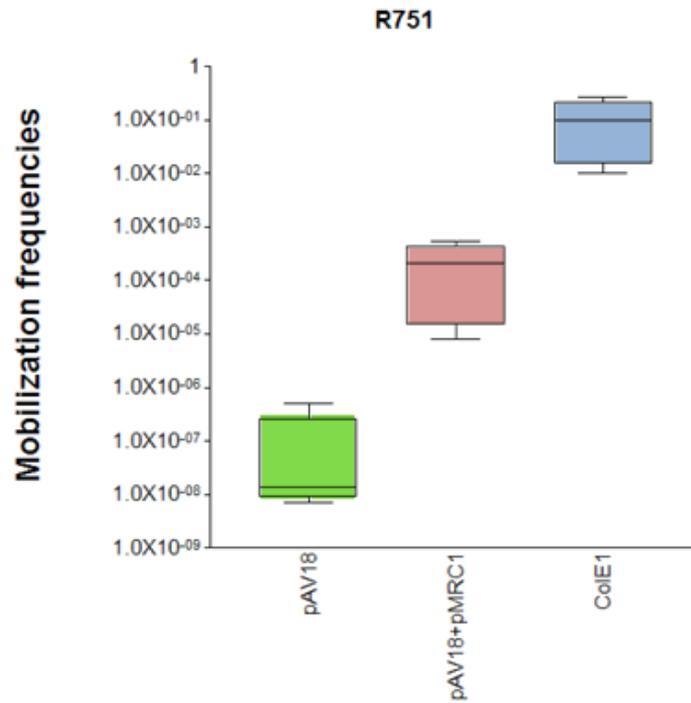
**Figure 11.** Mobilization frequencies using the pRL443 conjugative plasmid. Green: pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*]. Red: pMRC1 [wt-*mbeC* + wt-*mbeD*] + pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*]. Blue: wild-type ColE1 plasmid.

pAV18 is in the -5 order of magnitude, pAV18 + pMRC1 in the -1, and the wild type ColE1 in the 1.



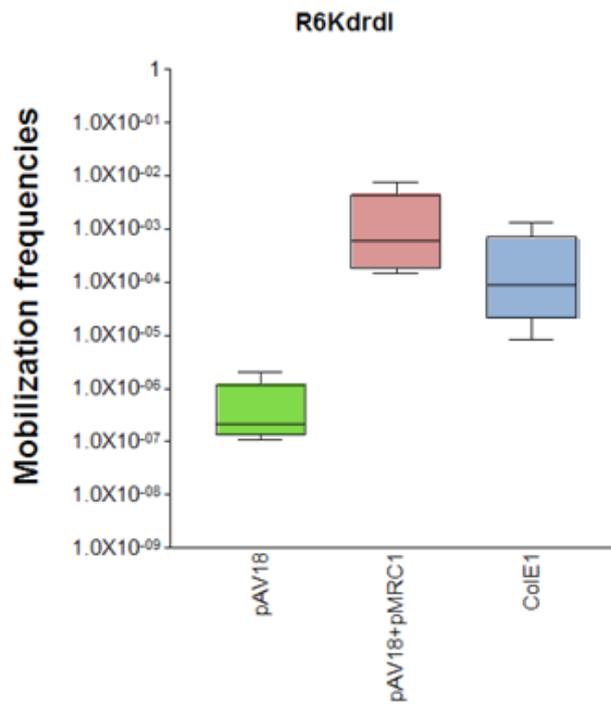
**Figure 12.** Mobilization frequencies using the R64-drd11 conjugative plasmid. Green: pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*]. Red: pMRC1 [wt-*mbeC* + wt-*mbeD*] + pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*]. Yellow: pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*] + pAV19 (*mbeB<sup>S</sup>*) + pAV23 (*mbeD<sup>S</sup>*). Blue: wild-type ColE1 plasmid.

pAV18 is in the -6 order of magnitude, pAV18 + pMRC1 in the -1, pAV18 + pAV19 + pAV23 in the -4 and the wild type ColE1 in the -1.



**Figure 13.** Mobilization frequencies using the R751 conjugative plasmid. Green: pAV18 [oriT-*mbeA<sup>S</sup>-mbeC<sup>S</sup>*]. Red: pMRC1 [wt-*mbeC* + wt-*mbeD*] + pAV18 [oriT-*mbeA<sup>S</sup>-mbeC<sup>S</sup>*]. Blue: wild-type ColE1 plasmid.

pAV18 is in the -7 order of magnitude, pAV18 + pMRC1 in the -3, and the wild type ColE1 in the -1.



**Figure 14.** Mobilization frequencies using the R6KdrdI conjugative plasmid. Green: pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*]. Red: pMRC1 [wt-*mbeC* + wt-*mbeD*] + pAV18 [oriT-*mbeA<sup>S</sup>*-*mbeC<sup>S</sup>*]. Blue: wild-type ColE1 plasmid.

pAV18 is in the -6 order of magnitude, pAV18 + pMRC1 in the -3, and the wild type ColE1 in the -4.

# Discussion

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CloE1 is historically one of the most well documented plasmids. Studies about its conjugation processes lasted for decades, and now that we are facing an antibiotic resistance threat a look back at it makes sense. What this study tries to convey is not only the need for useful and easy to manage tools in order to study plasmids and their conjugations methods but also make sure that these tools are functional and reproducible.

When we tried to insert the whole ColE1-MOB<sup>S</sup> region into cells we realized it was not possible. A variety of punctual mutations appeared along the sequence once it was sent for sequencing. Statistical analysis (data not published) determined that the number and location of these mutations was not significant, but they produced changes in the amino acid translation so it could not be used.

It was a pleasant surprise when different plasmids still produced the proteins necessary for mobilization. Nevertheless, *mbeB\** and *mbeC\** still could not be cloned in a single plasmid.

So we tried to do the same with the wild type versions of these genes. Somehow they can be cloned together but not their synthetic counterparts. However, early on in the experiments we noticed the synthetic plasmids were not complementing each other as well as they should be compared with the wild-type ones.

With the R388 conjugative plasmid there is an increase of one order of magnitude when using pMRC1 in comparison with only pAV18, it stays on the same order as the pAV18+pAV19+pAV23, and is still two orders of magnitude below the wild type conjugation frequencies. Plasmid R388 is not very effective at mobilizing the ColE1 plasmid as the other conjugative plasmids used in this study.

pRL443 conjugative plasmid makes a better job at mobilizing, with the wild-type at the 1 frequency, the pMRC1 following close behind and the pAV18 five orders of magnitude below. In this case, the *wt-mbeB + mbeD* genes make a real difference in the mobilization process.

The R64-drld11 conjugative plasmid manages to move the pMRC1 plasmid as well as the ColE1 plasmid. The three plasmid construct (pAV18 + pAV19 + pAV23) has a two order of magnitude improvement over just the pAV18. While *mbeB\** and *mbeD\** do not manage to compare with the effect of their wild type counterparts they still make some improvement in the conjugation.

For the R751 plasmid, the complementation between the synthetic genetic constructs makes an improvement over just pAV18 in four orders of magnitude, but is still two orders under the ColE1 frequency of conjugation.

R6K drdl 1 showed a slight increase in the frequency of pMRC1 over ColE1, still three orders above pAV18. This could be due to the inherent bias in the statistic method and would be balanced repeating the conjugation assays a few more times.

We theorized that the efficiency at mobilizing the ColE1 plasmid was related to the conjugative plasmid used, specifically in their Inc group but pR1443 and R751 have very similar Inc groups (P1 $\alpha$  and P1 $\beta$ , respectively) however, their ability to mobilize pMRC1 varies three to four orders of magnitude, which makes it unlikely.

Regardless, the question remains as of why the wild type *mbeB* + *mbeD* genes move more efficiently than their synthetic counterpart. Maybe being inside the *mbeA* gene, although in other reading frame, keeps some kind of regulation over them. It is also possible that during the construction of the synthetic version some RNA was lost or changes in the secondary structure of the protein made them lose effect.

In order to elucidate these questions, more research in the field needs to be conducted. The antibiotic resistance is already here, and while it cannot be beaten just with limitation or blockage of the conjugation, it could give us targets for future drugs which would act as cofactor of the actual antibiotics. Not only they would work stopping the conjugation processes while an antibiotic erases the actual infection, they could be used in antibiotic-producing plants to avoid the spreading of resistance and in biological waste, preventing the passing of these resistances to the environment.

# Conclusions

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- ❖ *mbeB* and *mbeD* are important players in the conjugation process since their involvement manages to improve conjugation rates several orders of magnitude in all the experiments conducted.
- ❖ The degree of complementation of pMRC1 (*mbeB* + *mbeD*) to mobilize pAV18 (*oriT-mbeC<sup>\*</sup>-mbeA<sup>\*</sup>*) depends on the conjugative plasmid used. Best functionalities were exhibited with plasmids pRL443, R64-drd11 and R6Kdrd1.
- ❖ Wild-type genes *mbeB* and *mbeD* (pMRC1) were more efficient at mobilizing the synthetic construction pAV18 than their synthetic version [pAV19 (*mbeB<sup>\*</sup>*) + pAV23 (*mbeD<sup>\*</sup>*)].
- ❖ Conjugation studies will provide targets for the inhibition of the conjugation that can be used to limit the spreading of antibiotic resistances.

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