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Detection of Horizontal Gene Transfer Mediated by Natural Conjugative Plasmids in *E. coli*

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Abstract

Conjugation represents one of the main mechanisms facilitating horizontal gene transfer in Gram-negative bacteria. This work describes methods for the study of the mobilization of naturally occurring conjugative plasmids, using two naturally-occurring plasmids as an example. These protocols rely on the differential presence of selectable markers in donor, recipient, and conjugative plasmid. Specifically, the methods described include 1) the identification of natural conjugative plasmids, 2) the quantification of conjugation rates in solid culture, and 3) the diagnostic detection of the antibiotic resistance genes and plasmid replicon types in transconjugant recipients by polymerase chain reaction (PCR). The protocols described here have been developed in the context of studying the evolutionary ecology of horizontal gene transfer, to screen for the presence of conjugative plasmids carrying antibiotic-resistance genes in bacteria found in the environment. The efficient transfer of conjugative plasmids observed in these experiments in culture highlights the biological relevance of conjugation as a mechanism promoting horizontal gene transfer in general and the spread of antibiotic resistance in particular.

Introduction

In 1946, Lederberg and Tatum[1] described a sexual process in *Escherichia coli* K-12 that is now known as conjugation. Bacterial conjugation is the process by which a bacterial cell (the donor) transfers unidirectionally genetic material to another cell (the recipient) by direct cell-to-cell contact. Conjugation is broadly distributed in bacteria[2,3], although the fraction of donor cells expressing the conjugation machinery is typically very small[4].

Plasmids are autonomously-replicating extrachromosomal DNA elements. In addition to genes involved in plasmid replication and maintenance, plasmids frequently carry a cargo of genes involved in adaptation to environmental challenges, such as heavy metals or exposure to antibiotics[5].

Conjugative plasmids are a class of plasmids with a set of specialized genes that allow their transfer to recipient cells and support their persistence following the transfer[6]. Conjugative plasmids vary in size from 21.8 kb to 1.35 Mb in bacteria in the phylum Pseudomonadota (synonymous with Proteobacteria), with the median around 100 kb[5,7]. They also generally have a low copy number, possibly to keep the metabolic burden on the host low[8,9].

The typical conjugative apparatus consists of four components: an origin of transfer (*oriT*), a relaxase, a type IV coupling protein, and a type IV secretion system (a tube-like structure called pilus that allows donors to contact recipients)[6]. Only a very small fraction of cells carrying conjugative plasmids express the conjugation machinery[4], but if the plasmids provide a fitness advantage, the transconjugants can rapidly expand in the population. Between 35% and over 80% of *E. coli* isolates collected from different habitats have conjugative plasmids with genes that confer resistance to at least one antibiotic[10,11]; therefore, horizontal gene transfer mediated by conjugative plasmids is a major mechanism driving the global spread of antibiotic resistance genes[12].

Mating experiments conducted in laboratory culture have shown that the conjugation frequency is affected by multiple factors, including the nature of the recipient cells, growth phase, cell density, donor-to-recipient ratio, whether conjugation is conducted in liquid or solid media, carbon, oxygen, bile salts, metal concentrations, presence of mammalian cells, temperature, pH, and mating time[13,14,15].

This work describes protocols to detect the presence of conjugative plasmids in a given host strain, to quantify the conjugation rates in solid culture, and to double-check their transfer to recipient cells. These protocols can be used as a first step for the identification of natural conjugative plasmids suitable for research. They use a minimum number of simplified steps because they are designed to screen the presence of conjugative plasmids in bacteria obtained from multiple sources (environmental, commensal, and pathogenic) at scale (dozens to hundreds of donors).

In addition, tests to detect whether the mobilization of a given conjugative plasmid is independent of the antibiotic used for detection (i.e., the relevance of the antibiotic resistance gene under selection) and to compare the conjugation rates of two conjugative plasmids found in different environmental isolates are shown.

Based on the genetic composition of the relevant conjugative plasmids (plasmid replicon and antibiotic resistance gene makeup), each step of the protocol can be modified to study the impact of a variety of factors likely to affect the conjugation rate.

General experimental design:

The essential components needed to set up a mating experiment are donor cells, a recipient strain, and solid media to select donors (antibiotic A), recipients (antibiotic B), and transconjugants (antibiotic A and B). Transconjugants are recipient cells that stably maintain the conjugative plasmid of the donor.

Donor cells are resistant to an antibiotic (antibiotic A) and are susceptible to the marker or markers used to select recipient cells (antibiotic B). The genomic location of the antibiotic resistance gene (i.e., whether it is located in the chromosome or a plasmid of the donor cell) does not have to be known *a priori*, because mobilization of antibiotic resistance markers to the recipient (after a direct donor-recipient contact) implies that the donor-provided markers were in a plasmid.

A recipient strain (known to take conjugative plasmids) needs to have a stable selectable marker not present in the donor; this selectable marker is generally resistant to an antibiotic or biocide located in the chromosome. The selection of the recipient to be used in mating experiments is critical, because some *E. coli* strains vary in their ability to take conjugative plasmids[16].

Once these components have been established, any colony that grows on media with both antibiotics (A and B) after a donor-recipient pair contact is a putative transconjugant (**Figure 1**). This is assuming that donors can grow in media with antibiotic A but cannot grow in media with antibiotic B, and that recipients are able to grow in media with antibiotic B, but not able to grow with antibiotic A. Transconjugation can be confirmed using two diagnostic tests. The first test consists of the detection (by polymerase chain reaction [PCR] amplification of genes, or other methods) of genes found in the conjugative plasmid in transconjugant colonies. The second test involves the use of differential colony color markers based on lactose metabolism. The differential colony color is revealed by the use of MacConkey agar; the lactose in the agar can be used as a fermentation source by lactose-fermenting (lac^+) microorganisms. These microorganisms produce organic acids, particularly lactic acid, which lower the pH. Neutral red is a pH indicator included in the media that turns from off-white to bright red/pink as the pH drops below 6.8[17]. Thus, *E. coli* lactose-positive strains produce larger pink colonies on MacConkey agar, whereas lactose-negative strains produce pale yellow and smaller colonies on MacConkey agar.

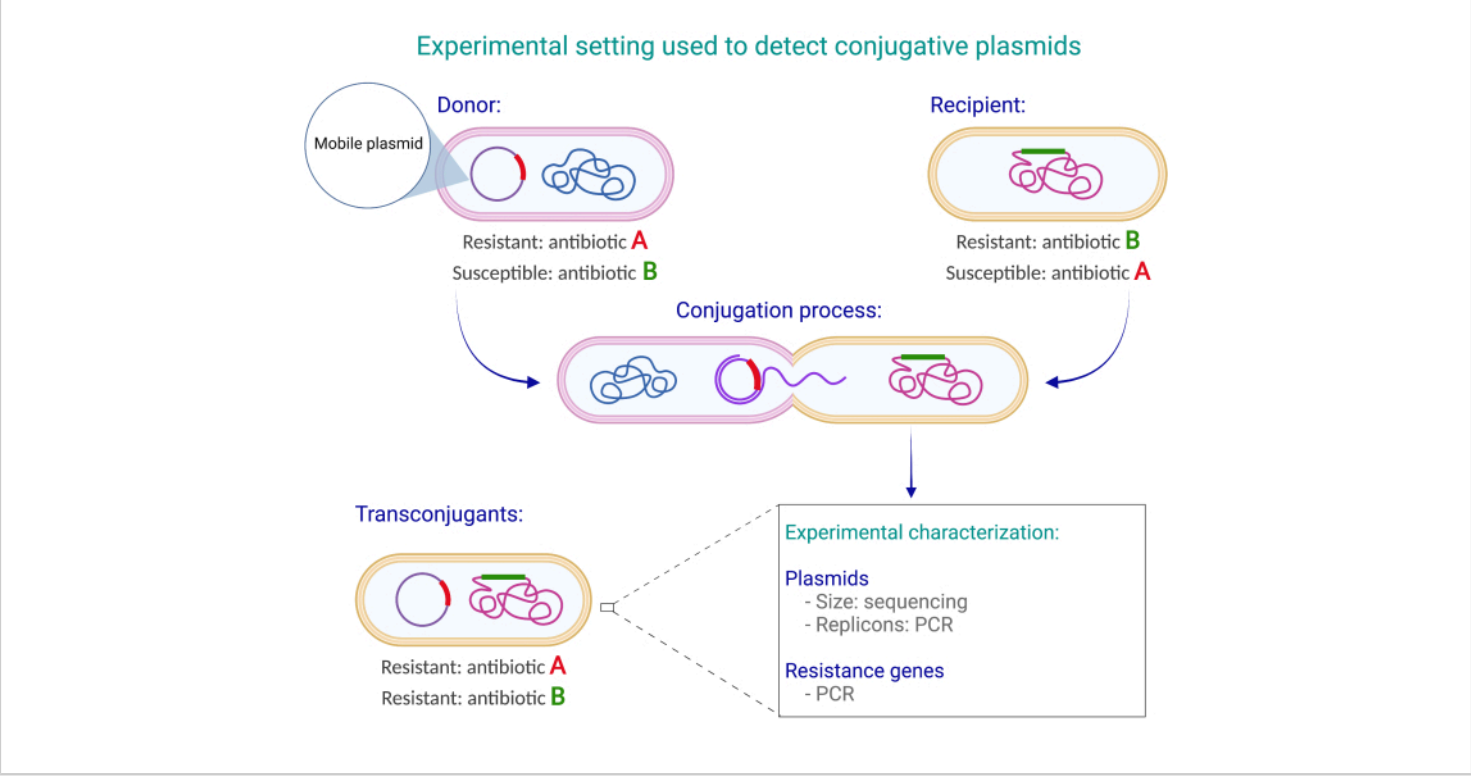

Figure 1: Experimental design used to detect the presence of conjugative plasmids in donor strains. In this example, the donor carries a conjugative plasmid with an antibiotic resistance gene that confers resistance to antibiotic A, but they

are susceptible to antibiotic B. Conversely, the recipient has a chromosomal resistance determinant that confers protection from antibiotic B, but it is susceptible to antibiotic A. Transconjugants are resistant to both antibiotics (A and B), because they have the conjugative plasmid of the donor that confers resistance to antibiotic A and the chromosome of the recipient that confers protection from antibiotic B. Please click here to view a larger version of this figure.

The conjugation rate for a donor-recipient pair (under given experimental conditions) can be calculated by dividing the number of transconjugants by the number of donors or by the number of recipients; the first rate indicates the fraction of donor cells exhibiting functional expression of the conjugation machinery by the donor[16,18], while the second rate indicates the ability of the recipient to take conjugative plasmids[19,20]. In this study, unless otherwise stated, the conjugation rate represents the fraction of recipient cells that become transconjugant (i.e., rate per recipient).

Here, two independent mating experiments are reported, involving one *E. coli* recipient and two *E. coli* donors. In addition, different antibiotics were used to select transconjugants for one of the donors to confirm that a single multidrug-resistant plasmid can be selected with any of the antibiotic resistance genes found in the conjugative plasmid.

The donor and recipient strains used in this work have been fully sequenced to understand all components of this experimental system; however, these protocols were designed to screen for the presence of conjugative plasmids in hosts of unknown sequence, and can be used in this experimental context as well; however, in this case, the relevant genes are sequenced first.

The donor and recipient strains used in the protocol are the following:

Donor 1. *E. coli* SW4955 was collected in a lake in Baton Rouge (LA, USA). It has a 134,797 bp conjugative plasmid (p134797) with IncFIC(FII) and IncFIB (AP001918) replicons. This conjugative plasmid has genes that confer resistance to third-generation cephalosporins ($bla_{CTX\text{-}M\text{-}55}$), aminoglycosides (*aac(3)-IIa* and *aadA1*), phenicols (*catA2*), tetracyclines (*tet(A)*), trimethoprim (*dfrA14*), and sulphonamides (*sul3*). For a complete map of p134797, please see **Figure 2A**. *E. coli* SW4955 is lactose-positive, producing pink colonies on MacConkey agar.

Donor 2. *E. coli* SW7037 was collected in Lake Erie (Ottawa County, OH, USA). It carries a 101,718 bp conjugative plasmid (p101718) with an IncI1-I(Alpha) replicon. This conjugative plasmid has a gene that confers resistance to beta-lactams ($bla_{CMY\text{-}2}$). For a complete map of p101718, please see **Figure 2B**. *E. coli* SW7037 is also lactose-positive, producing pink colonies on MacConkey agar.

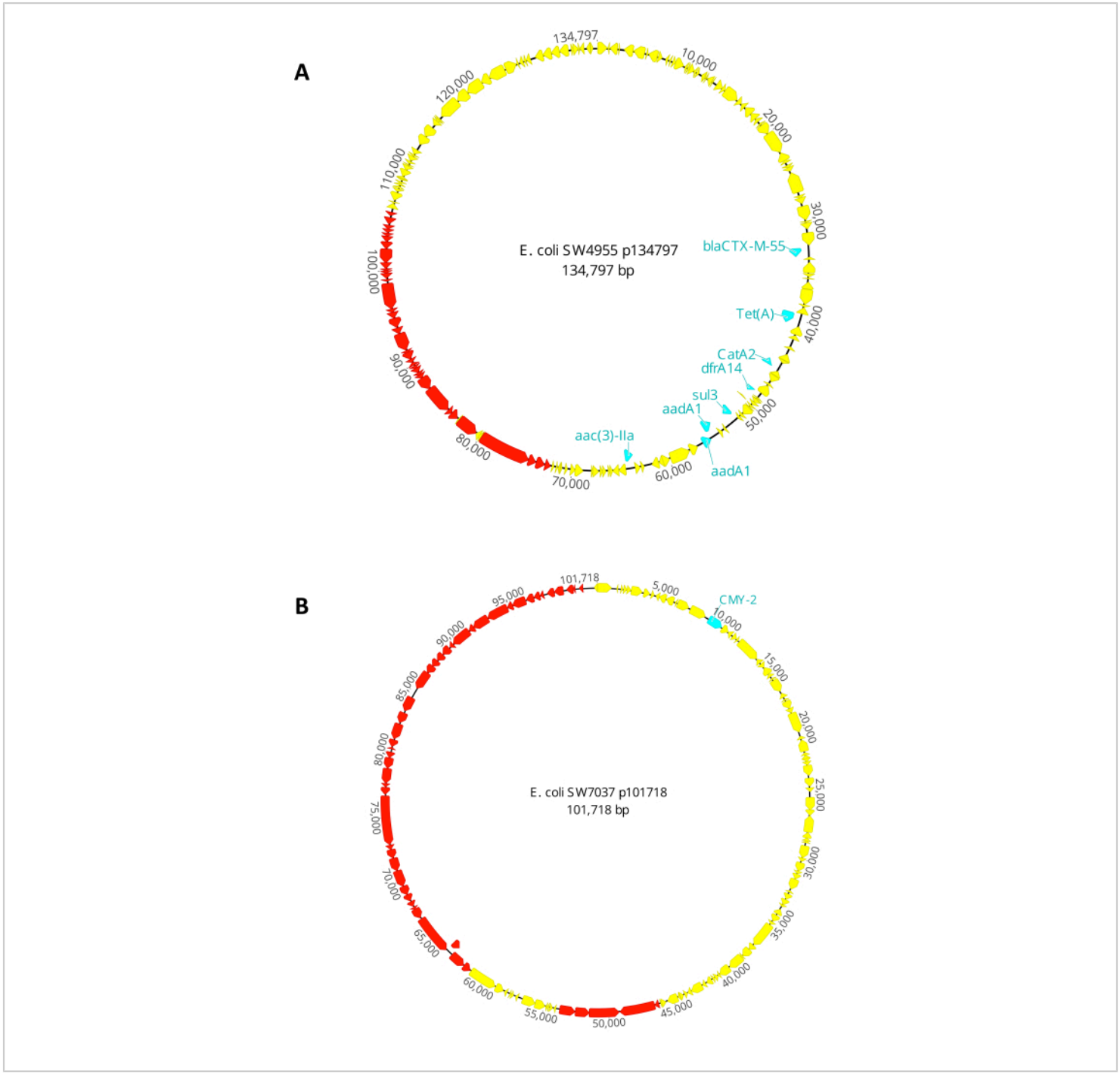

Figure 2: Genetic map of the conjugative plasmids used in this study. (**A**) Plasmid p134797, the conjugative plasmid found in *E. coli* strain SW4955. (**B**) Plasmid p101718, the conjugative plasmid found in *E. coli* strain SW7037. Antibiotic resistance genes are highlighted in blue, and genes belonging to the conjugative apparatus are highlighted in red. Please click here to view a larger version of this figure.

Recipient. *E. coli* LMB100 is used as recipient. This is a plasmidless strain that is resistant to rifampin (100 mg/L) and to streptomycin (100 mg/L). Having resistance to two antibiotics reduces the possibility of resistance mutations arising in the donor that would interfere with the interpretation of results. In addition, *E. coli* LMB100 is lactose-negative, and can be distinguished from the two donor strains because it produces pale yellow and small colonies (as opposed to larger, pink colonies) on MacConkey agar.

When the donor is lactose-negative, we recommend to use of a lactose-positive recipient (e.g., *E. coli* J53). The LMB100 and J53 strains are available to other labs for use. Please send a request to Dr. Gerardo Cortés-Cortés along with an address and FedEx number.

The solid media needed to select and count donors, recipients, and transconjugants is MacConkey agar in Petri dishes 100 mm in diameter. The addition of the following antibiotics is needed: (i) Media A: carbenicillin (100 mg/L) to count donors and to ensure that recipients cannot grow with this antibiotic. (ii) Media B: rifampin (100 mg/L) + streptomycin (100 mg/L) to count recipients and to ensure that donors cannot grow in these two antibiotics. (iii) Media AB: carbenicillin (100 mg/L) + rifampin (100 mg/L) + streptomycin (100 mg/L) to obtain and count transconjugants. (iv) Media C: no antibiotics to streak all isolates studied.

The conjugation rates of conjugative plasmids from *E. coli* SW4955 and *E. coli* SW7037 to *E. coli* LMB100 are compared. In addition, in the case of the p134797 conjugative plasmid (SW4955 strain), the antibiotic carbenicillin (100 mg/L) is replaced by gentamicin (2 mg/L), chloramphenicol (25 mg/L), tetracycline (10 mg/L), trimethoprim (20 mg/L), or sulfamethoxazole (100 mg/L) in subsequent experiments to establish if the antibiotic resistance marker used for selection has any impact on the results.

Protocol

1. Method 1: Conjugation

1. Day 1: Streak the donors and recipient. Streak separately from glycerol stocks on media C (MacConkey agar with no antibiotics), and incubate overnight at 37 °C.
   NOTE: This step is necessary to ensure that the experiment is conducted with pure isolates and to confirm the lactose phenotype by the color of colonies.
2. Day 2: Label a 14.0 mL culture tube for each donor and recipient. Select a single colony of each donor and recipient, and grow them overnight (18 h) in separate 14.0 mL culture tubes containing 2 mL of Mueller Hinton broth at 37 °C and shaking at 200 rpm.
   NOTE: In the strains used, the stationary phase is reached after overnight culture of 18 h.
3. Day 3: Measure the optical density at 600 nm (OD600) of the overnight culture of each donor and recipient (no vortexing) by using a 1:10 dilution of 900 µL of saline solution and 100 µL of the overnight culture.
4. Adjust the optical density of each donor and recipient to 2.0 (OD_{600} = 2.0) with sterilized saline solution (0.85% NaCl in water).
   NOTE: An overnight culture of *E. coli* with an OD_{600} = 2.0 has 1.6 x 10^9 CFU/mL.
5. Label a 1.5 mL microcentrifuge tube "mating tube", indicating the donor and recipient strains. Transfer 500 µL of the adjusted (OD_{600} = 2.0) suspension of each donor and recipient, and place them in the mating tube (this mating tube would have 0.8 x 10^9 CFU/mL of each

donor and recipient). Gently mix the mating tube by inversion.

6. Centrifuge the mating tube for 10 min at 500 x *g* at room temperature.

7. Without disturbing the pellet, pipette out 800 µL of the conjugation tube. There should be 200 µL left in the conjugation tube.
   NOTE: Discard the supernatant to a 10% bleach container to inactivate bacteria in the suspension.

8. Incubate the mating tube for 18 h (overnight) at 37 °C in an incubator. Mating occurs during the incubation time.
   NOTE: This step must be done without shaking in order not to break the conjugative pili.

9. As a negative control, streak the overnight culture of donors on media B and recipients on media A. Incubate the plates overnight at 37 °C

10. Day 4: Add 800 µL of saline solution to the mating tube and vortex to resuspend (this reconstitutes the mating mix to 1 mL).
    NOTE: Vortexing breaks the mating bacteria apart and homogenizes the culture to quantify the CFU/mL.

11. Prepare 1:10 dilutions (from 1 x 10^0 to 1 x 10^{-7}) of the mating mix. Plate 100 µL of dilutions 10^{-5} to 10^{-7} on media A and media B, and all dilutions on media AB.
    NOTE: Dlution 10^0 refers to the neat tube.

12. Allow the plates to dry before placing them in the incubator upside down. Incubate the plates for 18 h (overnight) at 37 °C.

13. Day 5: Inspect the negative controls to ensure that the streaks of pure overnight cultures of donors did not grow on media B, and the pure overnight culture of recipients did not grow on media A.

14. Record the number of colonies and dilutions that can be counted:
    1. Count the colonies in media A; these are the donors. The colonies should be pink (**Figure 3A,B**).
    2. Count the colonies in media B; these are the recipients and transconjugants. The colonies should be pale yellow (**Figure 3C**).
    3. Count the colonies in media AB; these are the transconjugants. The colonies should be pale yellow.
       NOTE: Select a countable plate. A countable plate has between 30 and 300 colonies (more than 300 colonies would be difficult to count, and less than 30 colonies are considered too small a sample size to present an accurate representation of the original sample).

15. Calculate the frequency of conjugation per donor or per recipient
    Frequency of conjugation per donor = (CFU/mL of the transconjugant [media AB])/ (CFU/mL of donors [media A]) x 100
    Frequency of conjugation per recipient = (CFU/mL of the transconjugant [media AB])/ (CFU/mL of recipients and transconjugants [media B]) x 100
    NOTE: The conjugation frequency for this protocol was calculated as transconjugants divided by the number of the recipient, multiplied by 100. According to the literature, the resulting quantity of conjugation could be named as exconjugant frequency, gene transfer frequency, conjugation frequency, recombinant yield, plasmid transfer efficiency, conjugation frequency based on the total bacterial count, proportion of transconjugants, fraction of transconjugants in recipient

population, transconjugant frequency, conjugation frequency, the logarithm of conjugation rate, transfer rate constant, conjugation rate per mating pair, conjugation coefficient, or conjugation efficiency[20]. This protocol refers to the term conjugation efficiency.

16. Store the transconjugants in glycerol stocks at -80 °C. Follow the substeps for preparing glycerol stocks.
    1. Select a single colony of transconjugants and grow them overnight (18 h) in 5.0 mL culture tubes containing 2 mL of Mueller Hinton broth supplemented with carbenicillin (100 mg/L), at 37 °C and shaking at 200 rpm.
    2. Label cryogenic vials, indicating the name of transconjugant, as follows: Tc + name of donor + antibiotic of selection in media A (as they are transconjugants, it is known that their background is the recipient strain, which is already resistant to streptomycin and rifampin but additionally harbors the plasmid carrying a beta-lactam resistance gene); for example, TcU1Carb. Add continuous numbers in case more than one transconjugant from the same donor need to be stored (e.g., TcU1Carb1, TcU1Carb2, etc.).
    3. Transfer 1 mL of the overnight culture to 1 mL of 50% glycerol (v/v; the final glycerol concentration should be 25%) and gently mix by inversion. Put the cryogenic vials on dry ice for 15 min and store the cryovials at -80 °C for future experiments.
17. For DNA extraction, streak the trasnconjugants from glycerol stocks on Mueller Hinton agar supplemented with carbenicillin (100 mg/L), and incubate overnight at 37 °C; then, follow recommendations from step 2.1.

2. Method 2: Polymerase chain reaction (PCR) to amplify antibiotic resistance genes and plasmid replicons in *E. coli* transconjugants

NOTE: Polymerase chain reaction (PCR) was developed by Dr. Kary Mullis in 1983. The PCR depends on specific primers (a short piece of DNA complementary to a given DNA sequence that acts as a point at which replication can proceed, generally 20-40 nucleotides in length and ideally with a guanine-cytosine content of 40%-60%), that are annealed to opposite strands of a denatured, double-stranded DNA template and extended through a thermostable DNA polymerase, thus generating an additional template for the next cycle of reactions, leading to the exponential amplification of the original template[21]. In this protocol, the replicons and resistance genes present in the conjugative plasmids were amplified to confirm the transfer in transconjugant recipient cells.

1. DNA extraction
    1. To detect the transfer of resistance genes carried by conjugative plasmids, use transconjugant DNA as a template. Use simple boil prep extraction to lyse the bacterial cell walls.

       NOTE: Simple boil prep extraction is a simple approach to lyse the bacterial cell walls. This extraction contains plasmid DNA mixed with genomic DNA, but as primers are designed according to specific DNA sequences of interest, this template is also useful for PCR. Plasmids can also be specifically purified, although yields tend to drop with increasing plasmid size[22]. This is a convenient stopping point. DNA can be stored for several months at -20 °C.
2. PCR

1. Given that the experiment has a negative and a positive control, it is beneficial to set up a pool for all the reactions in a 1.5 mL tube. Label a 1.5 mL tube as "pool" and the required PCR tubes with the name of the gene and sample (e.g., OXA-U1).

2. Pipette the PCR reagents in the following order into a 1.5 mL tube: sterile water, 2x Master Mix, primers, and polymerase (except the template DNA) (see **Table 1**). Gently mix by pipetting up and down at least 10 times. Keep on ice the entire time.
   NOTE: Wear gloves to avoid contaminating the reaction mixture or reagents. Try to avoid opening and mixing reagents and handling samples in the same area to prevent reagent contamination. Place the reagents into the ice bucket to suppress nuclease activity and nonspecific priming. Let them thaw completely before setting up a reaction. Keep the reagents on ice throughout the experiment. Taq Polymerase is added at the end because it is sensitive to pH changes; it needs to be buffered to avoid degradation or misfolding. The sequence of the primers is listed in **Table 2**-antibiotic resistance genes-and **Table 3**-replicons[23,24,25,26,27,28].

3. Add the template DNA to the corresponding tube. Avoid introducing bubbles and secure caps on the PCR tubes.

4. Place the PCR tubes into the thermal cycler.

5. Start the program. Refer to the program settings listed for each gene in **Table 2** (resistance genes) and **Table 3** (replicons).
   NOTE: Set PCR programs to hold the samples at 4 °C after the run.

   1. PCR conditions for replicons: one cycle of denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, elongation at 72 °C for 1 min, and a final extension of one cycle at 72 °C for 5 min.
      NOTE: These are the conditions standardized by Carattoli et al.[29].

6. When the program finishes, store the PCR tubes at 4 °C.
   NOTE: This is a convenient stopping point. PCR products can be stored for several months at -20 °C

7. Prepare a 1% agarose gel by weighing 0.6 g of agarose in a 250 mL flask and adding 60 mL of 1x Tris-acetate-EDTA (TAE) buffer (adjust reagents if a different gel size is needed). Melt the agarose carefully and slowly in a microwave to avoid agarose spilling over the flask, and let it cool down to approximately 55 °C (10-15 min).

   1. Prepare all the reagents using ultrapure deionized water and analytical grade reagents:

   2. TAE 50x Buffer (Tris base, acetic acid, and EDTA) (1 L): Mix 121.1 g of Tris base + 372.24 g of EDTA, and add 500 mL of distilled water. Dissolve with a magnetic stirrer. Add 57.1 mL of acetic acid, and add distilled water to a total volume of 1,000 mL.

   3. Autoclave at 15 psi, 121 °C for 15 min, and keep at room temperature until ready for up to 6 months.

   4. TAE 1x Buffer (1 L): Combine 20 mL of TAE 50x buffer with 980 mL of distilled water and mix.

8. Under a fume hood, add 6 µL of SYBR Green to the melted agarose, mix, and pour out into the tray (and comb), previously sealed with adhesive tape to avoid spillage. Let the gel set for 15-25 min until turbidity appears.
   NOTE: Other alternative dyes are also available[30,31,32,33].
9. Remove the adhesive tape and place the gel into the electrophoresis chamber, adding enough 1x TAE buffer to cover the gel.
10. Mix 5 µL of 6x DNA gel loading dye with 25 µL of each reaction. Gently mix by pipetting up and down at least 10 times.
    NOTE: Not all the PCR product needs to be loaded into the gel to visualize the expected product (adjust the amount of dye as corresponds). The remaining PCR sample could be saved and stored at -20 °C for several months.
11. Load the mix into wells (avoid introducing bubbles) and put the chamber lid down.
    NOTE: Load the first sample into the second well (reserve the first to the ladder), starting with the negative control.
12. Load 4 µL of 1 kb DNA ladder into the first well.
13. Run the electrophoresis at 120 V, 400 mA, and 60 min.
14. Visualize the gel under UV light (with a UV illuminator) and record the image.
    NOTE: If a PCR product is present, DNA bands stained can be detected using a standard UV transilluminator, a visible-light transilluminator, or a laser-based scanner.

Reagent	Stock Solution	Volume added to 50 µL reaction (1x)	Final Concentration	Example: volume added to 1x	Example: volume added to 3x (pool)	Example: volume added to each tube Final Vol. 50 µL	Volume added to positive control	Volume added to negative control
Sterile water	-	21.5 µL (q.s. to 50 µL)	-	21.5 µL	64.5 µL	49 µL/tube	49 µL/tube	49 µL/tube
Master Mix	2x	25 µL	1x	25 µL	75 µL			
Forward Primer	25 µM	1 µL	0.5 µM	1 µL	3 µL			
Reverse Primer	25 µM	1 µL	0.5 µM	1 µL	3 µL			
Template DNA	Variable (100–200 ng/µL)	Variable (1 µL)	Variable	0.5 µL	1.5 µL			
Polymerase	5 Units/µL	0.5 µL	2.5 Units	-	-	1 µL (transconjugant)	1 µL (donor)	1 µL (recipient)

NOTE: q.s. is a Latin abbreviation for quantum satis meaning the amount that is needed.

Table 1: PCR reagents and pool for three reactions (an example).

Primers (sequence listed in the 5' - 3' orientation)	PCR program	Expected size (bp)[Ref.]
***bla*$_{CTX-M}$ group 1**	94 °C 7 min	(864)[23]
CTX-M: GGTTAAAAAATCACTGCGYC	94 °C 50 s (35 cycles)	
CTX-M: TTGGTGACGATTTTAGCCGC	50 °C 40 s	
	68 °C 1 min	
	68 °C 5 min	
***bla*$_{CMY-2}$**	95 °C 3 min	(1855)[24]
CMY-2-F: GATTCCTTGGACTCTTCAG	95 °C 30 s (30 cycles)	
CMY-2-R: TAAAACCAGGTTCCCAGATAGC	53 °C 30 s	
	72 °C 30 s	
	72 °C 3 min	
aac(3')-II	94 °C 5 min	(237)[25]
aac(3')-II-F: ACTGTGATGGGATACGCGTC	94 °C 30 s (32 cycles)	
aac(3')-II-R: CTCCGTCAGCGTTTCAGCTA	60 °C 45 s	
	72 °C 2 min	
	72 °C 8 min	
aadA	94 °C 5 min	(283)[26]
aadA1: GCAGCGCAATGACATTCTTG	94 °C 1 min (35 cycles)	
aadA2: ATCCTTCGGCGCGATTTTG	60 °C 1 min	
	72 °C 1 min	
	72 °C 8 min	
sul-3	94 °C 5 min	(799)[27]
sul-3-F: GAGCAAGATTTTTGGAATCG	94 °C 1 min (30 cycles)	

sul-3-R: CATCTGCAGCTAACCTAGGGCTTTGGA	51 °C 1 min	
	72 °C 1 min	
	72 °C 5 min	
***tet*(A)**	95 °C 5 min	(957)[28]
TETA-1: GTAATTCTGAGCACTGTCGC	95 °C 30 s (23 cycles)	
TETA-2: CTGCCTGGACAACATTGCTT	62 °C 30 s	
	72 °C 45 s	
	72 °C 7 min	
***dfr*A**	95 °C 5 min	(302) This Work
dfrA-F: CATACCCTGGTCCGCGAAAG	95 °C 1 min (30 cycles)	
	55 °C 1 min	
dfrA-R: CGATGTCGATCGTCGATAAGTG	72 °C 1 min	
	72 °C 7 min	
catA2	95 °C 5 min	
catA2-F: GACCCGGTCTTTACTGTCTTTC	95 °C 1 min (25 cycles)	(225) This work
catA2-R: TCCGGTGATATTCAGATTAAAT	60 °C 1 min	
	72 °C 1 min	
	72 °C 7 min	

Table 2: Primers and PCR programs used to amplify diagnostic resistance genes.

Replicon	Primer (sequence listed in the 5' - 3' orientation)	Target	PCR program	Expected size (bp)
IncFIB	F: TCTGTTTATTCTTTTACTGTCCAC	repA	94 °C 5 min	683
	R: CTCCCGTCGCTTCAGGGCATT		94 °C 1 min (30 cycles)	
			60 °C 30 s	
IncFIC	F: GTGAACTGGCAGATGAGGAAGG	repA2	72 °C 1 min	262
	R: TTCTCCTCGTCGCCAAACTAGAT		72 °C 5 min	
IncI1	F: CGAAAGCCGGACGGCAGAA	RNAI		139
	R: TCGTCGTTCCGCCAAGTTCGT			

Table 3: Primers and PCR program used to classify plasmids p134797 and p101718 by PCR-based replicon typing[29].

Representative Results

Given that donors SW4955 and SW7037 have been sequenced, these two donor strains are expected to have the resistance phenotypes corresponding to the resistance gene profile identified in their genomic sequence. Plasmid p134797 (from SW4955) has genes that confer resistance to third-generation cephalosporins (*bla*$_{CTX-M-55}$), and resistance to aminoglycosides (*aac(3)-IIa* and *aadA1*), phenicols (*catA2*), tetracyclines (*tet(A)*), trimethoprim (*dfrA14*), and sulphonamides (*sul3*); no antibiotic resistance genes were found in the SW4955 chromosome. Plasmid p101718 (from SW7037) only carries one antibiotic resistance gene (*bla*$_{CMY-2}$), expected to confer resistance to third-generation cephalosporins (*bla*$_{CTX-M-55}$). Again no antibiotic resistance genes were found in the SW7037 chromosome.

Following mating, detection of the transfer of the two conjugative plasmids bearing all the resistance genes mentioned above was expected. Positive conjugation detection implies that the recipients identified as transconjugants should have acquired the conjugative plasmids. The presence of the diagnostic resistance genes for the conjugative plasmids in the transconjugants (as detected by PCR) was also expected.

To illustrate the methods described here, the ability of two *E. coli* environmental isolates to transfer plasmid DNA by conjugation was tested. A schematic representation of the experimental setting is shown in **Figure 1**. Two donors (*E. coli* SW4955 and SW7037) and one recipient (*E. coli* LMB100) were mated independently. The gene map of conjugative plasmids found in *E. coli* SW4955 (p134797) and SW7037 (p101718) is shown in **Figure 2**.

Conjugative plasmids were selected with carbenicillin and counter-selected using rifampin and streptomycin, whose resistance determinants are in the chromosome of the

recipient. MacConkey agar was used to distinguish the lactose-positive donors (which produce large, pink colonies) from the recipient and transconjugants (which are lactose negative and produce smaller, pale yellow colonies) (**Figure 3**).

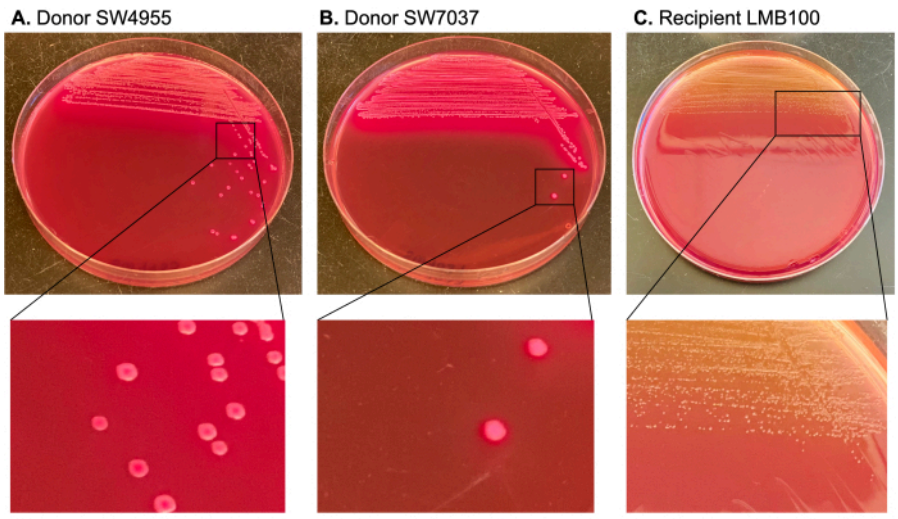

Figure 3: Illustration of the differential color markers for donor and recipient colonies on MacConkey agar. The colonies corresponding to donors are pink on MacConkey agar because they are lactose positive. This distinguishes donor from recipient colonies, which are pale yellow and smaller (lactose negative). Please click here to view a larger version of this figure.

The mobilization of the conjugative plasmid p134797 was detected, with each of the five antibiotics corresponding to the five different classes of resistance genes found in the plasmid.

An example of how to calculate the conjugation efficiency (CE) for strain SW4955 is presented. From the assay with strain SW4955, 172 CFU of transconjugants were counted in the plate from dilution 10^{-2}; the count of the recipient from the corresponding dilution (10^{-2}) was 10,300,000 CFU/mL (**Table 4**).

The CE is calculated as follows:

CE = (174 CFU/mL)/(10,300,000 CFU/mL)

CE= 1.67 x 10^{-5} transconjugants/recipient

	LMB100		Transconjugants from strain SW4955 selected with Carbelicillin	Conjugation Efficiency
Dilution	CFU (countable plate)	Approx. CFU/mL	CFU (countable plate)	

10^{0}	confluent	1.03E+09		
10^{-1}	confluent	1.03E+08		
10^{-2}	confluent	**10300000**	**172**	**1.67 x 10^{-5} transconjugants/ recipient**
10^{-3}	confluent	1030000		
10^{-4}	confluent	103000		
10^{-5}	confluent	10300		
10^{-6}	confluent	1030		
10^{-7}	**103**	103		
Values used to calculate the CE are highlighted in bold.				

Table 4: An example of how to calculate the conjugation efficiency (CE) for strain SW4955.

The results are shown in **Table 5**, expressed as conjugation efficiency per recipient. The five conjugation efficiencies obtained using strain SW4955 as a donor were all within the same order of magnitude. These results indicate that the mobilization of the conjugative plasmid can be detected regardless of the selection used for transconjugant identification.

Using the strain SW7037 as a donor, the conjugation efficiency obtained was three orders of magnitude lower; these results allow the comparison of conjugation efficiencies of different donors and plasmid types with the same recipients.

	Conjugation efficiency				
Strain	Carbenicillin (100 mg/L)	Gentamicin (2 mg/L)	Chloramphenicol (25 mg/L)	Tetracycline (10 mg/L)	Trimethoprim (20 mg/L)
SW4955	1.67 x 10^{-5}	5.67 x 10^{-5}	2.17 x 10^{-5}	7.62 x 10^{-5}	1.36 x 10^{-5}

SW7037	2.14×10^{-6}				

Table 5: Conjugation efficiencies of *E. coli* donor strains SW4955 and SW7037 depending on the antibiotic used for selection.

In transconjugants, the presence of replicons and all the resistance genes encoded in two conjugative plasmids tested, and their corresponding replicons, was checked by PCR. The conditions used for these diagnostic PCR reactions are shown in **Table 3**.

The diagnostic gels are shown in **Figure 4**. These gels confirm the presence of the expected replicons in transconjugants (IncFIC and IncFIB in p1347975, and IncI1 in p101718). They also confirm the presence of the expected antibiotic resistance genes, namely the $bla_{\text{CTX-M-55}}$ group (beta-lactam), *aac(3)-II* and *aadA* (aminoglycoside), *catA2* (phenicol), *tet(A)* (tetracycline), *dfrA14* (trimethoprim), and *sul3* (sulphonamide) for p1347975, and $bla_{\text{CMY-2}}$ for the p101718 (**Figure 4**).

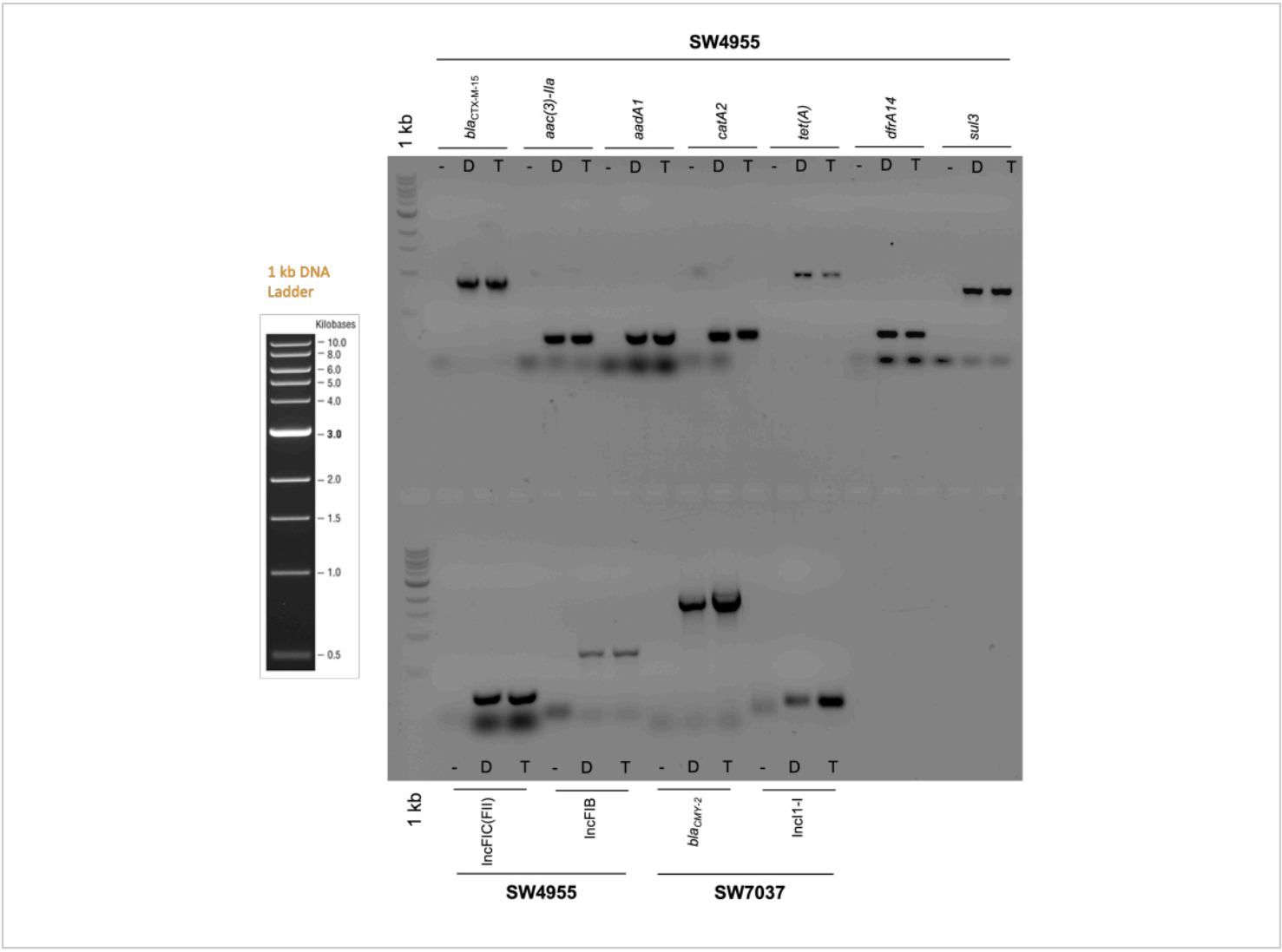

Figure 4: Diagnostic electrophoresis gels. Gel electrophoresis of PCR products of antibiotic resistance genes and plasmid replicons found in conjugative plasmids p134797 and p101718 in the donor cells (strains SW4955 and SW7037, respectively) and in the corresponding LMB100 transconjugants. Carbenicillin was used for plasmid selection. Abbreviations. -: negative control (DNA of strain LMB100 was used as a negative control); D: donor; T: transconjugant. Expected amplicon sizes: $bla_{CTX-M-55}$: 864 pb; *aac(3)-IIa*: 237 bp; *aadA1*: 283 bp; *catA2:* 225 bp*; tet(A)*: 957 bp; *dfrA14*: 302 bp; *sul3*: 799 bp; IncFIC(FII): 262 bp; IncFIB: 683 bp; bla_{CMY-2}: 1,855 bp; IncI1-I: 139 bp. The gel has 1% agarose. Please click here to view a larger version of this figure.

Discussion

Conjugative plasmids provide access to a communal pool of genes within a particular environmental setting through recombination and horizontal gene transfer[34]. Thus, conjugative plasmids are evolutionary entities capable of acquiring and conferring functions that allow bacteria to adapt to multiple conditions (including resistance to antibiotics,

resistance to metals, acquisition of metals, biofilm formation, and pathogenic genes, among others) within a temporal scale of hours.

This work presents a protocol for the identification of conjugative plasmids in bacteria. For the protocol to work, the markers used need to differentiate donor and recipient strains, as shown by the controls in media A and B. They also need to effectively select cells carrying the plasmid. The mating reaction is critical. In this reaction, donors and recipients need to make prolonged contact (through pili) for conjugation to occur. Anything that can disrupt cell-to-cell contact, such as an insufficient incubation time or shaking or stirring the culture, thus decreases the efficiency of conjugation. The choice of recipient is also critical, as some recipient strains are refractory to conjugation[35,36]. LMB100 is proposed as the recipient of choice, as it is able to take plasmids of multiple incompatibility types.

The conjugation rate is specific for each plasmid-donor chromosome pair, recipient, and environmental condition. The conjugation of specific plasmids has been found to be sensitive to a large number of variables, including growth phase, cell density, donor-to-recipient ratio, whether conjugation is conducted in liquid or solid media, carbon, oxygen, bile salts, metal concentrations, presence of mammalian cells, temperature, pH, and mating time[13,14,15]. The study of these interactions depends on the initial detection of a conjugative plasmid to be studied in depth. Thus, the protocol described here can also be modified to explore the impact of different experimental variables, although this comes at the cost of restricting the number of screened donors. It can also identify mobilizable plasmids in hosts that have helper plasmids (i.e., plasmids that enable conjugation by providing functions missing from the mobilizable plasmids *in trans*[6]).

Knowing the sequence of the conjugative plasmid is recommended (but not necessary to detect conjugation, as discussed above). When the donors are lactose-negative (producing yellow colonies on MacConkey agar), a lactose-positive recipient (producing pink colonies on MacConkey agar) can be used to distinguish true transconjugants from donor cells that are able to grow on the media to select transconjugants. The protocol described here is designed to detect conjugative plasmids from environmental, commensal, and pathogenic members of the family Enterobacteriaceae; however, it can be used with any bacterial species with a suitable donor, recipient, antibiotic(s), and color markers. The identification of these critical components in other bacteria requires systematic studies using multiple donors, recipients, and markers (antibiotics and colors). This protocol has not been tested in Gram-positive bacteria.

Several variants of the method presented here have been reported in the literature, recently reviewed[20]. The qualitative outcome can also be referred to in different ways (e.g., exconjugant frequency and gene transfer frequency), and dimensionless units can be used to report conjugation efficiency (mL/CFU x h)[20].

The protocol described here solves several limitations previously not addressed by existing methods[16,18,19,20]. First, a suitable recipient has been identified. Second, the use of two antibiotics (rifampin and streptomycin) to select true transconjugants minimizes the possibility that donors evolve resistance to one of the antibiotics used to select transconjugants through spontaneous mutagenesis. False transconjugants can also result from bystander protection by hydrolysis of the antibiotic used to select transconjugants

by enzymes (e.g., beta-lactamases) produced in large quantities by the donor. Third, several experimental controls are included to ensure that the product of mating is a true transconjugant (i.e., a recipient cell that has stably incorporated the conjugative plasmid of the donor). Here, we presented two independent methods to test the authenticity of the transconjugants, namely a colorimetric marker in MacConkey agar, and PCR detection of the replicons and antibiotic resistance genes of the conjugative plasmid in the recipient. Also, the protocol described here is designed to isolate and characterize conjugative plasmids from environmental, commensal, and pathogenic members of the family Enterobacteriaceae (*Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Salmonella*, *Shigella*, and other species).

To understand the mechanics of conjugation, experimental observations have been modeled using computer simulations. These predictive models estimate the frequency of plasmid transfer for given growth densities of donors, recipients, and transconjugants. A model known as the endpoint model found thresholds above which the rate of plasmid transfer in liquid culture, and concluded that the transfer rate is unaffected by cell density, donor:recipient ratio, and mating time[19]. Fluorescence *in situ* hybridization (FISH) has been used as an alternative method of transconjugant plasmid detection. FISH allows plasmid visualization using fluorescence microscopy through the hybridization of a DNA probe and a target DNA. Thus, FISH allows the visual detection of plasmid flows across different cell populations[37], although it does not have the same level of sensitivity as the method presented here if transconjugants are detected by visual screening as opposed to selection.

There is an enormous need to understand the biology of conjugative plasmids that are dispersing antibiotic resistance genes through different components of the ecosystem (clinic, agriculture, sewage, wildlife, domestic animals, soils, rivers, and lakes). In sum, the simplified experimental conditions presented in the protocols described here facilitate the screening of donors at scale, and thus represent a key tool for the study of horizontal gene transfer originating in conjugative plasmids from a variety of sources. They can be used to investigate the prevalence of antibiotic-resistance genes or other clinically-relevant genes in conjugative plasmids from multiple sources and bacteria. They can also be adapted for the study of conjugation *in vivo* (e.g., in the gut of vertebrates) and to study the conditions that modulate the efficiency of conjugation. All these studies will greatly add to understanding how the mobilization of multidrug-resistant conjugative plasmids contributes to the spread of multidrug resistance.

Disclosures

The authors have nothing to disclose.

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