

# **FREQUENCY-DEPENDENT TRANSMISSION IN PLASMID CONJUGATION**

Jorge Rodriguez Grande, Fernando de la Cruz Calahorra

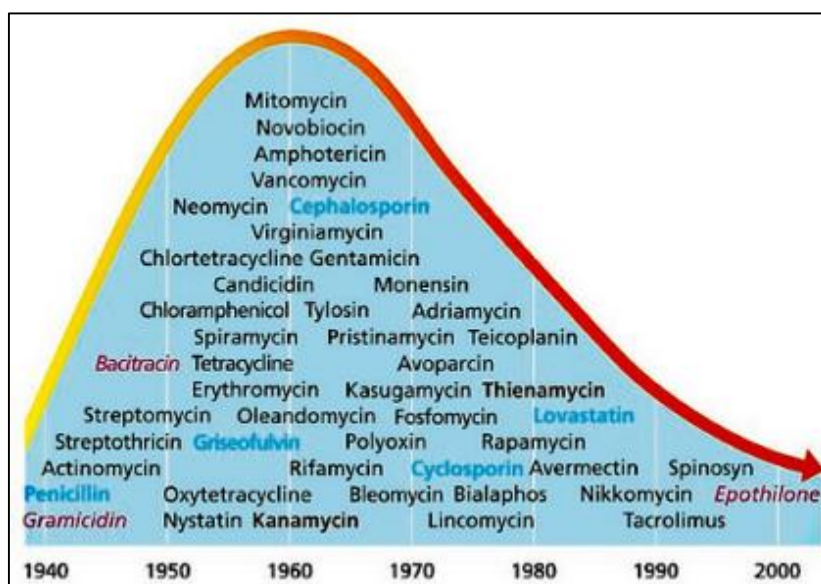
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# INTRODUCTION

Pharmaceutical treatment against bacterial infections started two thousand years ago: chinese, egyptian and greek healers used different kinds of mold to treat infected wounds. Description of an antibiotic phenomenon over *B. anthracis* was first made by Koch and Pasteur in 1877, the first drug specific against bacteria, “Salvarsan”, was developed by Ehrlich in 1907. Finally, the specific agents generated by *Penicillium notatum* were first described by Fleming as “penicillin” (although some voices raised the decade before pointing to C. Picado as the actual discoverer, in 1915-1927) and isolated in 1940 by Florey, This drug was produced at industrial scale altogether with gramicidin during the Second World War by the allies. Germany had developed in 1932 the first sulfonamide, “Prontosil” by a team led by Nobel Prize Gerhard Domagk.



**Fig1. New antibiotic discovery through XX century.** Several factors such as limitation on the natural repertory of antibiotic drugs, FDA regulations, “disenchantment” from the pharmaceutical industry and resistance emergence have slowed research.

Since then huge efforts have been put in the discovery of new antibiotics, as can be seen in **Fig.1** but research on new antibiotics supposes an ever increasing cost that pharmaceutical companies are no longer willing to take as long as benefits do not outweigh costs. Furthermore, antibiotic resistances -a

phenomenon already described with the discovery of penicillinase in 1940 (1) - emerge continuously, limiting the time that new drugs remain useful.

The use, misuse, underuse and overuse of antibiotic for 60 years not only in medical environments but also in daylife, and in non-therapeutical uses in agriculture and animals (prohibition was recommended as early as in 60’s (2) have produced the huge spread of these toxic agents all over the world that probably will give rise to a post-antibiotic new era.

Antibiotic presence in the environment imposes a selective pressure on bacteria that leads to positive selection of resistance genes. Nowadays, over 20.000 potential genes have been described or predicted to have resistance functions in genomic studies (3). The most important reservoir of multidrug-resistant strains of bacteria, fundamentally enteric Gram (-) bacteria, is the mammalian –mostly human and livestock animals – gut. From there, multi-resistant strains spread to wastewaters and rivers to the environment.

**Historic overview.**-Antibiotic resistance genes were likely to exist before the first antibiotic was isolated and commercialized, but they were limited to sub-populations while there was no selective pressure to maintain them. However, only a decade after the first antibiotic drug was proven, the first resistant strains of coli had appeared (1). By 1959, horizontal gene transfer was pointed to be playing a major role in the spreading of this features, (features that in fact were devastating the after-Second World War Japan due to multidrug resistant strains of *Shigella dysenteriae*.(4) This information was welcomed cold and skeptically in western countries as Japanese research lacked some prestige at that time. (5).

Nowadays horizontal gene transfer (HGT) is globally acknowledged as the major player on the spreading of antibiotic resistance genes: although point mutations also might contribute in some extent, the highly versatile and broad host range of HGT makes it a great tool to share adaptive specific responses only in specific situations while minimizing the total burden of carrying additional genetic material for the global population.

Thus, HGT is thought to be responsible for a huge contribution in all prokaryotes, and there are several works pointing that horizontal transfer in eukaryotic genomes has been an important force propelling genomic variation and biological innovation. Only about 200 cases documented of HGT have been reported in multicellular eukaryotes; most cases involving transposable elements. Not only are TEs the single most abundant entity of large eukaryotic genomes (e.g. about half of the human genome and 85% of the maize genome), they are also one of their most dynamic components. The reason for their presence in eukaryotic genomes in spite of the decrease in fitness would be their ability to horizontal transfer (6).

There are **three mechanisms of Horizontal gene transfer**: conjugation, transduction and transformation (7). The first one is the most abundant in nature, at least in terms of number of species affected and cross-species mobility. Plasmid conjugation requires contact between a donor bacteria and a recipient one and is mediated by a mating pair protein machinery known as 'pilus' that injects DNA into the recipient. There have been reported plasmid conjugations between different strains of the same bacterial species, but also between different species of bacteria, or even between bacteria and eukaryotes and archaea (8). Bacterial conjugation in Gram positive

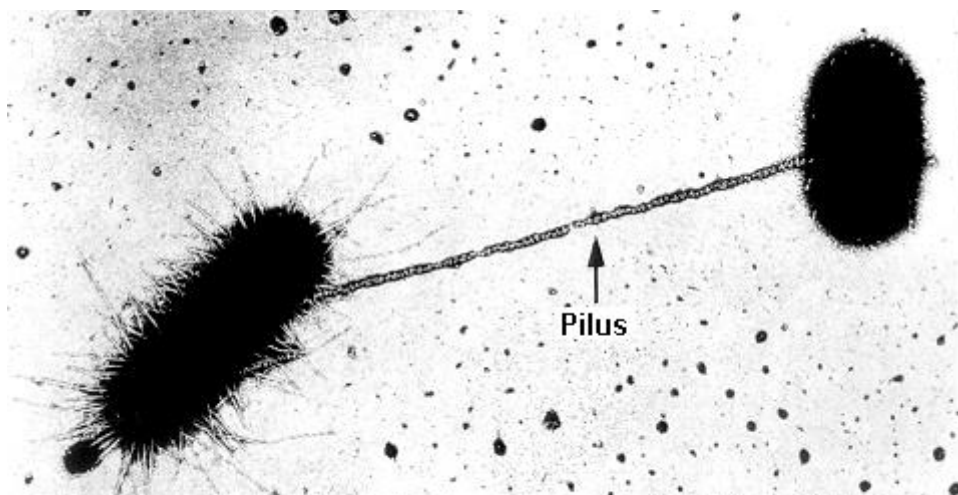
bacteria is different: it is mediated by direct contact and no mating pair formation pilus is needed. The first cases reported in Gram positive (23) were induced by a diffusible protein factor allegorically called “pheromone”, as long as conjugation was considered in those years the equivalent of sex in animals, and all the vocabulary was referred to that concept. All references hereafter to conjugation are to conjugation in gram – bacteria.

Up to date, a huge number of mobilizable and conjugative plasmids have been described. No estimation exists on the proportion of plasmids that are conjugative, mobilizable, or nontransmissible. Even if there are over 1,700 complete plasmid genomes in GenBank, there is experimental evidence of transmissibility for just a small fraction of them. Also, there are other genetic elements transmissible by conjugation, that are known as ICEs (Integrative and Conjugative Elements), non-replicative elements that are integrated into the chromosomal DNA after conjugation, and that can be excised from there to be conjugated as single strand DNA. Recent studies are bridging the gap between plasmids and ICEs as equivalent features are being described in both of them.

Conjugation in gram – bacteria is allowed by three groups of DNA sequences:

The first one is the *oriT*, a target sequence recognized by the mobilization machinery. Plasmids bearing this short sequence (up to 500bp), enter a three-step process that is catalized by the relaxosome, which melts the double strand, cleaves DNA at the *nic* site and binds covalently a central protein to the new 5' end and carry the single strand to the recipient cell (9).

The second group is the mobilization machinery, which builds up the relaxosome. This relaxosome recognizes the *oriT* sequence and attaches 5' end to a specific tyrosine of the relaxase, forming a nucleoprotein that unwinds the double strand and is transported to the new recipient



*Fig. 2. Mating pair formation, as seen by electron microscopy.*

cell. A T4 Coupling Protein is required for this nucleoprotein to be recruited and transported to the channel in the conjugative pilus.

The third group is the “mating pair formation” or “conjugative pilus”, a Type IV Secretion System (10) consisting of a huge secretory complex spanning the two membranes, with a multimeric pilus which establishes contact with the recipient cell and that can be either rigid or flexible, depending on the plasmid (see Fig.2).

Using computational methods, the entire population of known plasmids have been classified in groups by their conjugative protein systems or their mobilization proteins. All the mobilizable plasmids are classified into six relaxase families; these plasmids make a half of the total population. Among them, only a half are also conjugative, and they are grouped into four families by their Type IV Secretion System. Divergent evolution has been observed between these two groups of conjugative and mobilization genes, the latter having evolved together with the Type 4 Coupling Proteins. Phylogenetic patterns of mobility proteins are consistent with the phylogeny of the host prokaryotes, suggesting a common evolutionary history.

Coevolution of host and plasmids has been studied in some reviews. When a plasmid colonizes a bacterial host for a long time (i.e., time >400 generations), it decreases the host fitness for a while, and cost often is reversed by some advantage like antibiotic resistance. But as coevolution goes on it finally improves the bacterial fitness, showing a clear signature of coevolution between mutualist organisms at different levels. (11)

Genetic information flows at high rates among prokaryotes, leading bacteria and archaea to have a small core genome that is conserved within a species and a large pangenome that is highly variable leads to the integration of new genetic information, creating large functional leaps that allow fast adaptation to new environments or to stressful conditions.

Despite gene flow between plasmids and chromosomes, genes for certain functions show strong tendencies to occur on plasmids while others consistently occur on chromosomes. Functions generally associated with plasmids are diverse, but all are useful only in locally restricted contexts; it is argued that the selective consequences of the greater horizontal (within generation) transmission of plasmids are responsible for this pattern (12). The apparent trends in eukaryote plasmids and transposons to lack these same characters also accords with predictions of the local adaptation hypothesis, because genes on these genetic units are generally no more horizontally mobile than chromosomal genes. There are theoretical reasons to expect that plasmid genes tend to evolve more rapidly than chromosomal genes.

Several strategies have been used to limit bacterial conjugation and thus, avoid antibiotic resistance genes to spread: inhibition of the relaxase of F plasmid in *E. coli* (13), inhibition of the conjugative relaxase of plasmid pCU1, inhibition of conjugation by phages (14), and by fatty acids (15).

Epidemiologic approaches were applied long time ago to plasmid spreading, looking at plasmids as parasites infecting bacterial hosts (16). Mathematical models in epidemiology use  $R_0$  as the infectivity of a plasmid. (17).  $R_0$  is essentially the number of successfully offspring a parasite or parasitic infection is capable of producing in the absence of density-dependent constraints. For plasmids is the average number of conjugations produced when one infected individual is introduced into a wholly susceptible host population. Infectious process can be modelled as:

$$R_0 = \frac{\beta \cdot N}{(\alpha + b + v)}$$

With  $\beta$ ,  $\alpha$ ,  $b$ ,  $v$  and  $N$  being respectively: transmission rate, host mortality, death rate in absence of infection, recovery rate of infected hosts and total population. With some variations, this is the basis for conjugation modelling.

$R_0$  plays a central role in determining the likelihoods of establishment, persistence and spreads after introduction. A high transmission potential greatly facilitates establishment and spread, but it may not enhance persistence if the parasite is a major cause of mortality or generates immunity in hosts that recover. In that cases, populations show oscillatory behavior, with the risk of that parasite will become extinct during the low phase, after an epidemic, being that risk accentuate with higher transmission potentials, as long as oscillations become more pronounced.

**Density dependent and frequency dependent models.** The basic assumption upon which mathematical models are built is that conjugation resembles an infectious process where, given a contact between a receptor cell (R) with a plasmid-containing cell (donors D and transconjugants T), there is a certain probability that the receptor cell receives the conjugative plasmid (becomes “infected”). Under these premises, the conjugation rate equals the number of receptor cells times the rate at which they contact other cells in the population, times the probability of that contact being infective. The contact rate times the probability of infection given a contact is named in classical epidemiological theory the Force of Infection ( $\lambda$ ) and follows:

$$\lambda = r_e \cdot P_I \cdot P_T$$

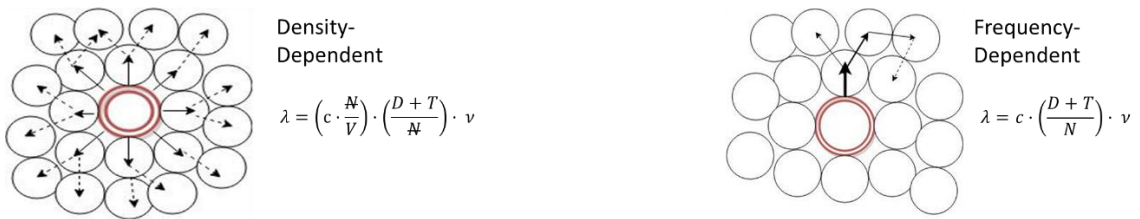
Where  $r_e$  is the rate of encounters per cell,  $P_i$  is the probability of that encounter being with an "infected" cell (D or T),  $P_T$  is the probability that, given an encounter between an R cell and a D (or a T) cell, transmission occurs: this probability of conjugation given a contact with an infected is assumed to be constant and to depend on the specific infectivity of the agent considered. Therefore, the rate at which new transconjugants T are formed is just:

$$\frac{dT}{dt} = \lambda \cdot R$$

Since  $dT/dt$  has units of  $\text{cells} \cdot \text{time}^{-1}$  and  $R$  has units of cells,  $\lambda$  must have units of  $\text{time}^{-1}$ .

Density dependent transmission (**DDT**) assumes that contacts depend on the concentration (or density per unit area) of individuals. In most cases, this dependence is assumed be linear. The contact rate under density-dependent transmission is assumed to be a linear function of the cell concentration,  $r_e = c \cdot N/V$  (or cell density where  $r_e = c \cdot N/A$ ). Models incorporating density-dependent disease transmission functions generally provide a good fit for airborne and directly transmitted bacterial or viral diseases (17).

Frequency-dependent transmission (**FDT**) is generally used to describe infectious processes where the rate of contacts per susceptible individual is relatively independent from the density. Here, "relatively independent" means that the number of contacts can be assumed to be roughly constant along a significant range of possible cell densities. Sexually-transmitted diseases are usually modeled by frequency-dependent transmission, since the average number of sexual contacts that an individual has does not usually depend on densities. The contact rate under FDT is assumed to be constant and known as  $c_f$ .



Previous works (18) demonstrated that incorporation of frequency dependent disease transmission eliminated a host density threshold for disease increase and made coexistence between host and pathogen more difficult. Coexistence in frequency-dependent systems requires regulation by some external density-dependent factor (e.g., increased mortality rates due to limited resources at high density). If there is no host recovery and no reproduction by diseased individuals, then



coexistence further requires that the degree of density dependence be different in the healthy and diseased classes (17).

Parasites like plasmids are favored to exploit their hosts prudently to prolong infection, increase horizontal transmission and avoid killing the host. They can take two evolutionary phenotypes: to keep prudent exploitation or to reproduce and spread rapidly.

When only one parasite inhabits a host the best strategy to improve persistence is the first one. However, when other competitors appear, the faster they exploit the host the bigger the fitness they get, leading them to “choose” the second option and exhausting rapidly the shared resources. Abundance of hosts/richness of the environment and competition among parasites (plasmids in our case) are the two motors in this evolutionary dynamics.

As time passes by, two differentiated scenarios emerge: competition among genetically related parasites and poor environments, leading to limited virulence; and strong competition among non-related competitors in rich environments, leading to prevalence of the most virulent.

Transmission is directly related to parasite fitness, showing the inverse fashion to virulence. The consensus solution for optimal prevalence is to keep low virulence, and rise the horizontal spread only in high density of hosts. Virulence models (19) pointed to a decreased vertical transmission (decreased stability in plasmids) in these cases, extreme confirmed experimentally in (20) and (21)

This equilibrium could bring an explanation to the self-repressing profile of many plasmids (15) (22) that inhibit their activity after entrance in a host, decreasing the burden on the host once persistence is assured.

This trade-off, when applied to recipient-plasmid systems, means that the loss of fitness associated to plasmid carriage, has to be necessarily compensated by horizontal spreading. This concept is central to the studies of epidemiology and plasmid transmission. Finally, it might be useful for us when using highly transmissible plasmids as tools for stopping antibiotic resistance spreading.



## BACKGROUND

Bacterial conjugation had been studied quantitatively before. Studies have been done on conjugation in silico, in vitro (25), and in vivo (26).

Also, big efforts have been put in studying conjugation in a structural way: Type 4 Secretion Systems used by conjugative plasmids have been finely described (10), as long as it has been the relaxosome of many plasmids (9).

Many methods have been used to quantify conjugation efficiency and many different units for rates of conjugation have been used (27).

A vast majority of the work in this topic of bacterial conjugation and its role in the ecology of plasmid spreading in populations of bacteria are based on a seminal work from Levin et al (16). In this paper, a density-dependent transmission model was stated for the kinetics of plasmid spreading as derived from a simple mass-action model.

Measurements on conjugation efficiency are often confusing (27) when walking through bibliography: almost each author or group of authors uses a particular measurement unit. Setting aside enzymological approaches (28), some experiments used units related to  $\beta$  (transmission rate),  $R_0$  (infectivity per donor cell per generation time), and  $\gamma$  (transmission probability given a contact, known as end-point method (29), while other results were relative to transconjugant production. Moreover, conjugation rates are sensitive to chemical, physical and biological conditions, and this condition is usually neglected when measuring or modelling plasmid conjugation and resistance genes spreading systems.

## OBJECTIVES

The focus of this work is to reach a framework that allows us to characterize dynamics of plasmid spreading, carrying antibiotic resistance genes. With this goal in mind, an in vitro model that tries to be as similar as possible to the biological systems in which antibiotic resistance spreading happens will be used. The final objective is to measure the essential features: rates of conjugation, rates of replication, etc. to build a model scalable to ecological systems. Ultimately, it should bring help to predict the spreading of antibiotic resistance genes and to develop strategies to stop them.

Conjugation is a complex process regulated at multi-scale levels: from transcription to protein degradation there are many key processes hard to quantify that module the efficiency.

However, as conjugation effects can be easily measured and quantified, and as long as its fundamental components have been known and described for a long time, it could be a good system for quantitative biological engineering and systems biology. Moreover, the conjugation machinery is thought to be restricted to a few genes, and even less regulators, which will ease the work.

With this in mind a series of experiments aiming to improve conjugation efficiency by up- or down-regulating some components of the conjugation system were set up. The objective is to modulate at will the efficiency of conjugation, propelling it when necessary and limiting it when not. It could help to build computational systems based on plasmids as wires and conjugation as signals.

## MATERIALS & METHODS

**Bacterial strains and plasmids.**- *Escherichia coli* was used as organism for all experiments. Bw27783 strains [ $\Delta(\text{araD-araB})$ ,  $\Delta\text{lacZ}$ ,  $\lambda^-$ ,  $\Delta(\text{araH-araF})$ ,  $\Delta\text{araE}$ ,  $\Delta(\text{rhaD-rhaB})$ ] were used for both donors and recipients. Donor strains carried chromosomal resistance gene for nalidixic acid ( $\text{Nal}^R$ ), while recipients carried chromosomal resistance for rifampicin ( $\text{Rif}^R$ ).

R388 (Trimethoprim resistant) plasmid was used as plasmid model in all experiments otherwise stated. R388 is a ~34 kb auto-conjugative plasmid from the IncW family, these plasmids only conjugate in solid surfaces. The other auto-conjugative plasmid is pOX38, a derivative of F plasmid, used for conjugation in liquid medium.

Antibiotics were used, when appropriate, at the following concentrations: Nalidixic acid ( $25 \mu\text{g}\cdot\text{ml}^{-1}$ ), rifampicin ( $50 \mu\text{g}\cdot\text{ml}^{-1}$ ), Trimethoprim ( $20 \mu\text{g}\cdot\text{ml}^{-1}$ ), Chloramphenicol ( $25 \mu\text{g}\cdot\text{ml}^{-1}$ ).

**Construction of pBAD33::TrwABC.**- A DNA segment of about 4.8 kb containing three ORFs, namely TrwA, TrwB and TrwC, from R388 was amplified by PCR with Phusion DNA polymerase. PCR primers were designed to bear Hind III and KpnI restriction sites. This product was inserted into the plasmid pBAD33 (“Tight Regulation, Modulation, and High-Level Expression by Vectors Containing the Arabinose pBAD Promoter” Guzman et al. (July 1995) *Journal of Bacteriology* p.4121-4130) by HindIII/KpnI digestion and ligation with T4 DNA ligase. The resulting plasmid construct had the arabinose regulable promotor pBAD upstream the TrwABC sequence, lacking the regulator sequences for trwA. Thus, this operon is not auto-repressed.

**Complementations.**- All the R388 mutants were kindly donated by the lab staff. Complementations were build up from competent *E. coli* Bw27783 ( $\text{Nx}^R$ ) at exponential growth; competence status was achieved by sequential steps of centrifuging and resuspending with distilled (Elix) water at  $4^\circ\text{C}$ . Plasmids were introduced by electroporation with a BioRad microPulser. Transformed cultures were diluted in 1 ml. of LB at  $37^\circ\text{C}$  and grew for 1-3 hours before plating in the corresponding antibiotic. Validation of positive colonies was made by PCR with different primers than those used when creating the clone.

**Conjugation assays.-** The conjugation experiments were performed over 24-well plates filled with solid LB-agar. Mating combinations were mixed right before plating avoiding occasional liquid conjugations. 10 ml. of donor and recipient cultures, both in stationary phase, were centrifuged and resuspended in LB in order to cleanse antibiotic from the medium. Optical density at 600nm. was measured with a density cell meter (Ultrospec 10 from Amerham Bioscience). Bacterial cultures were concentrated 10x (except for the dilution experiments) and mixed at different proportions, based on the OD measurements. 15  $\mu$ l of the mating mix was plated on the LB-agar wells.

Mating experiments were always performed at 37°C. Conjugations were stopped at exact times (conjugation is an exponential phenomenon and slight deviations in mating time produce big differences in transconjugant formation) using 1 ml of Phosphate Buffer Saline 1x.

Donor, recipient (+transconjugant) and transconjugant populations were plated in Petri plates filled with LB-agar containing the appropriate antibiotics, previously diluting them with PBS in order to obtain populations between 30 and 300 colonies per Petri plate.

**Secondary conjugation elimination.-** Conjugation experiments were performed in the same way as described before, but two donor strain were built:

- the first one carrying the mobilizable plasmid pSU4910 (pSU18::oriT) and conjugative but non-mobilizable pAP711 (R388 $\Delta$ oriT) (Demarre G. “A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncP $\alpha$ ) conjugative machineries and their cognate Escherichia coli host strains” et al. *Research in Microbiology* 156 (2005) 245–255) .
- The second donor strain carried the mating pair formation operon inserted in the chromosome, and the DNA transmission region in a plasmid (pBAD33::oriT-TrwABC). Both kindly provided by lab colleagues.

### **Mathematical models for plasmid spreading.-**

In epidemiology, the so-called “Force of Infection” (FOI or  $\lambda$ ) is used to measure infectivity of a pathogen.

- **r** : rate of encounter per cell

- **Pi**: Probability of encounter being between donor and receptor. The probability is the frequency of plasmid-carriers in the population:  $\left(\frac{D+T}{N}\right)$
- **Pt**: Probability of that encounter resulting in transmission. ( $\nu$ )

$$\lambda = P_i \cdot P_t \cdot r \text{ (eq.1)}$$

Variation on Force of Infection ( $\lambda$ ) was studied and contrasted with the two models, studying the formation of transconjugants in a period of time lacking of replicative growth:

$$\frac{dT}{dt} = \lambda \cdot N_{\text{rec}} \text{ (eq. 2)}$$

This Force of Infection depends on the total population only in DDT models.

The total transconjugant formation (in a given period of time) depends linearly on population in FDT models:

$$\lambda = c \cdot \left(\frac{D+T}{N}\right) \cdot \nu ; \text{ (eq.3a)}$$

and in a quadratic way in DDT models:  $\lambda = \left(c \cdot \frac{N}{\nu}\right) \cdot \left(\frac{D+T}{N}\right) \cdot \nu \text{ (eq. 3b).}$

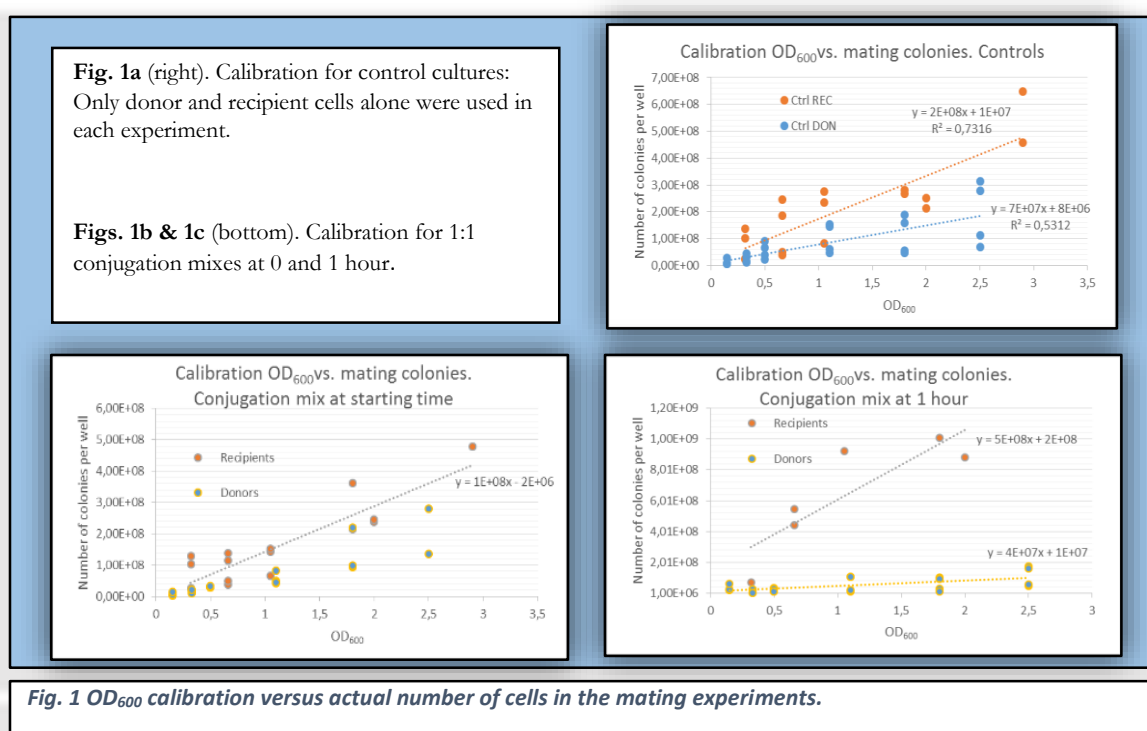
Discrimination between the two models was made by plotting normalized concentration of transconjugants (concentration of transconjugants in one experiment divided by the maximum concentration of transconjugants in any of the dilution experiments) versus the normalized concentration of total population. As shown before in eq. 2., dependence of the transconjugant generation on total population can follow a linear or a quadratic relationship. As both [Tc] and [N] axis are in logarithmic scale, this will be traduced in slope=1 or slope=2 respectively.

# RESULTS

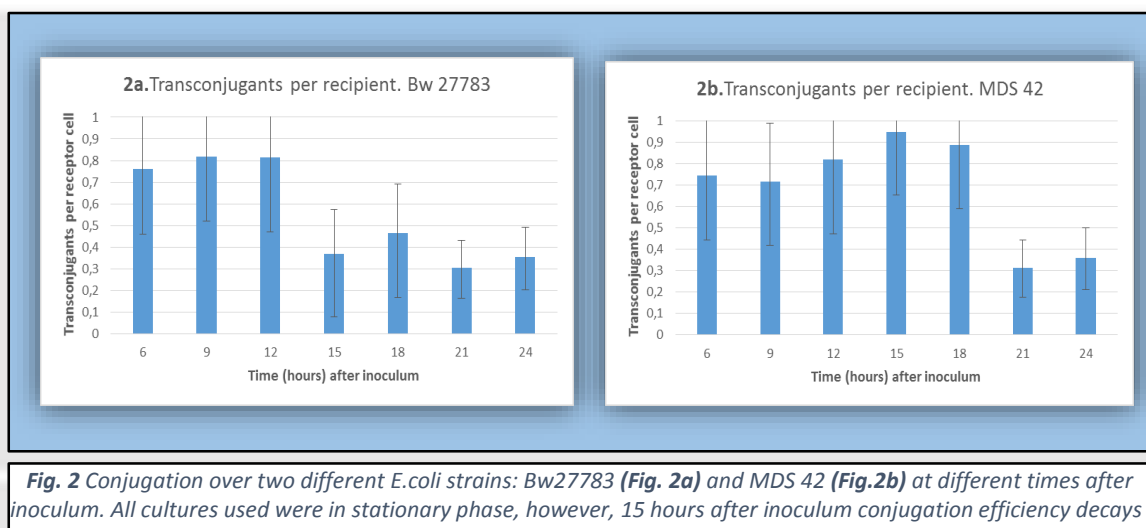
## PART I. Characterization

### Conjugation setup

Conjugation on E.coli (Nal<sup>R</sup>) carrying the R388 plasmid (Tp<sup>R</sup>) was performed over E.coli (Rif<sup>R</sup>) recipient cells at 1:1 proportion expressed as Donor: Receptor.



Bacterial cultures were grown for variable periods of time and fixed at different OD<sub>600</sub> in order to calibrate real numbers of cells in the conjugation well. Recipient cell cultures seemed to be more populated at same optical densities

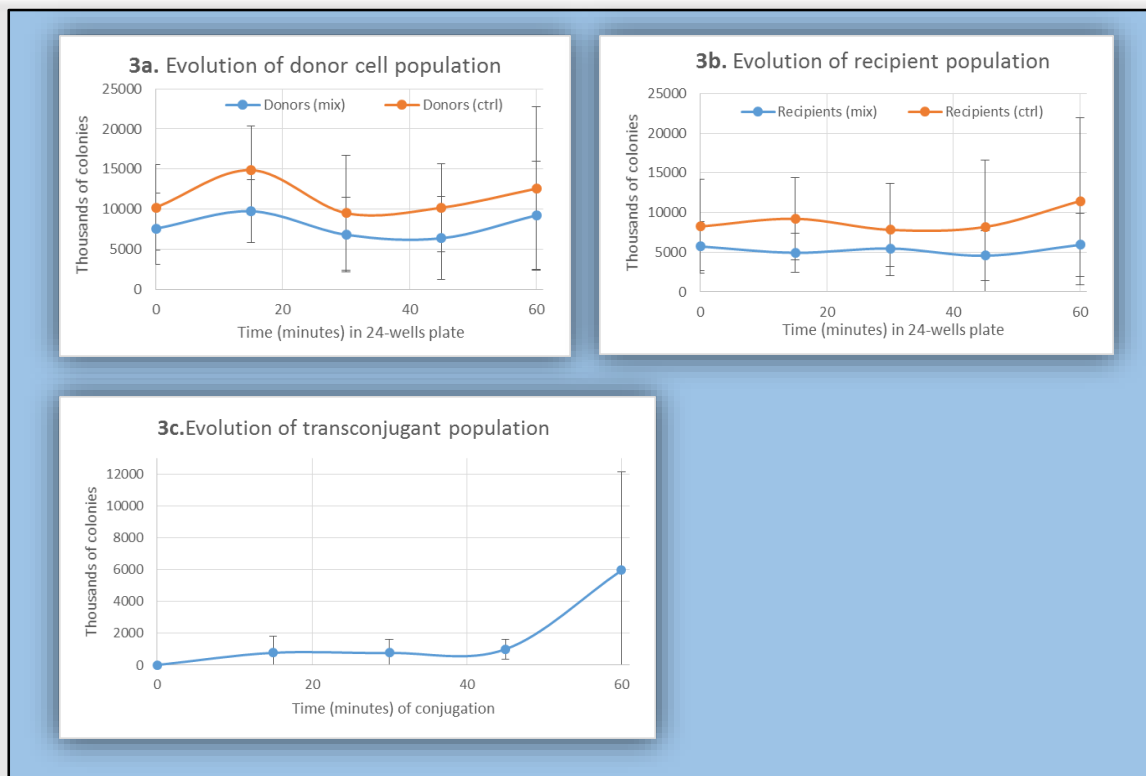


Bacterial cultures were grown for variable periods of time in stationary phase and conjugation was performed for 1 hour. Bw 27783 and also MDS 42 were tested as recipient cells, both presenting chromosomal rifampycin resistance genes. Donor cells used are Bw27783 (Nal<sup>R</sup>) R388<sup>+</sup> as usual. In one hour it is produced less than one transconjugant per recipient cell. Besides that, conjugation decreased slightly in cultures grown longer than 18 hours.

### Conjugation kinetics

Conjugation may proceed gradually, making the plasmid to spread continuously in the population. Nonetheless, it is possible for it to expand by explosive leaps instead if it takes some time since the contact donor: receptor starts until the conjugation process is fulfilled. To test this, mating formation was stopped at 0', 15', 30', 45' and 60' after mixing in the LB-agar well. Some transconjugants appear almost as soon as the droplet gets dry in the LB-agar well (0-2 minutes) and this population grows slowly for 30-40 minutes experiencing a rapid burst at 40-60 minutes.



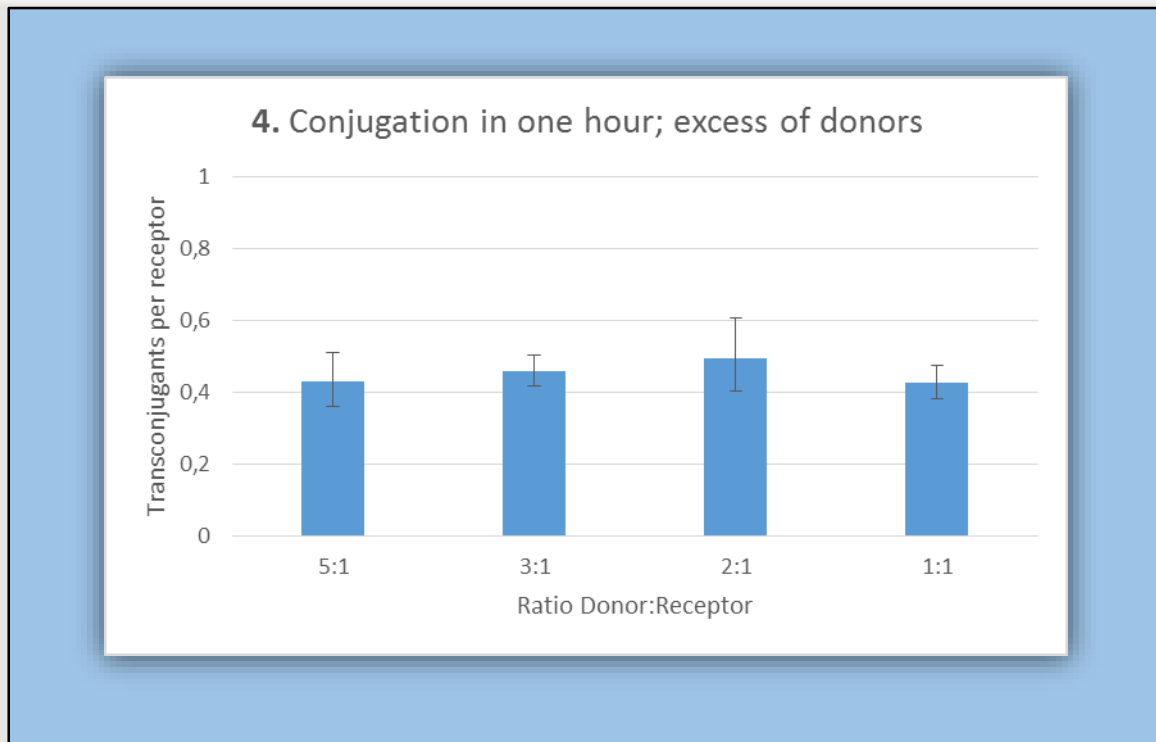


**Fig. 3. Population growth and Conjugation dynamics of R388.** In figs. 3a and 3b donor and recipient populations were measured for 1 hour each 15 minutes. Control populations (with no mating) showed equivalent dynamic fashion for 1 hour. In fig.3 the dynamics of transconjugant population for 1 hour is showed.

No growth was observed for recipient nor donor cells in these periods of time.

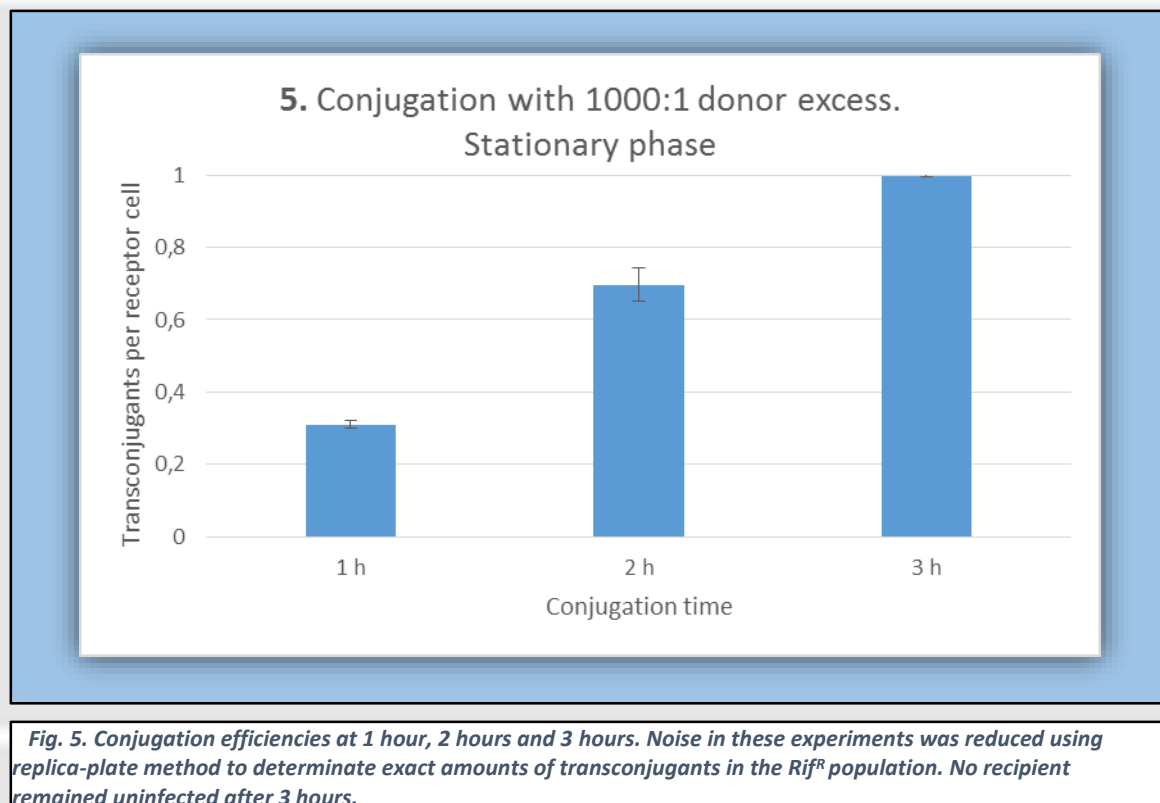
### Conjugation limits

In order to know if there exists a limit on conjugation efficiency or in conjugation time imposed by the recipient populations, or if there is a conjugation-inhibited subpopulation of recipients, we increased gradually the amount of donors using the same amount of recipients (see **Fig.4**). For conjugations 1 hour long, the amount of transconjugants formed did not depend on the amount of donors:

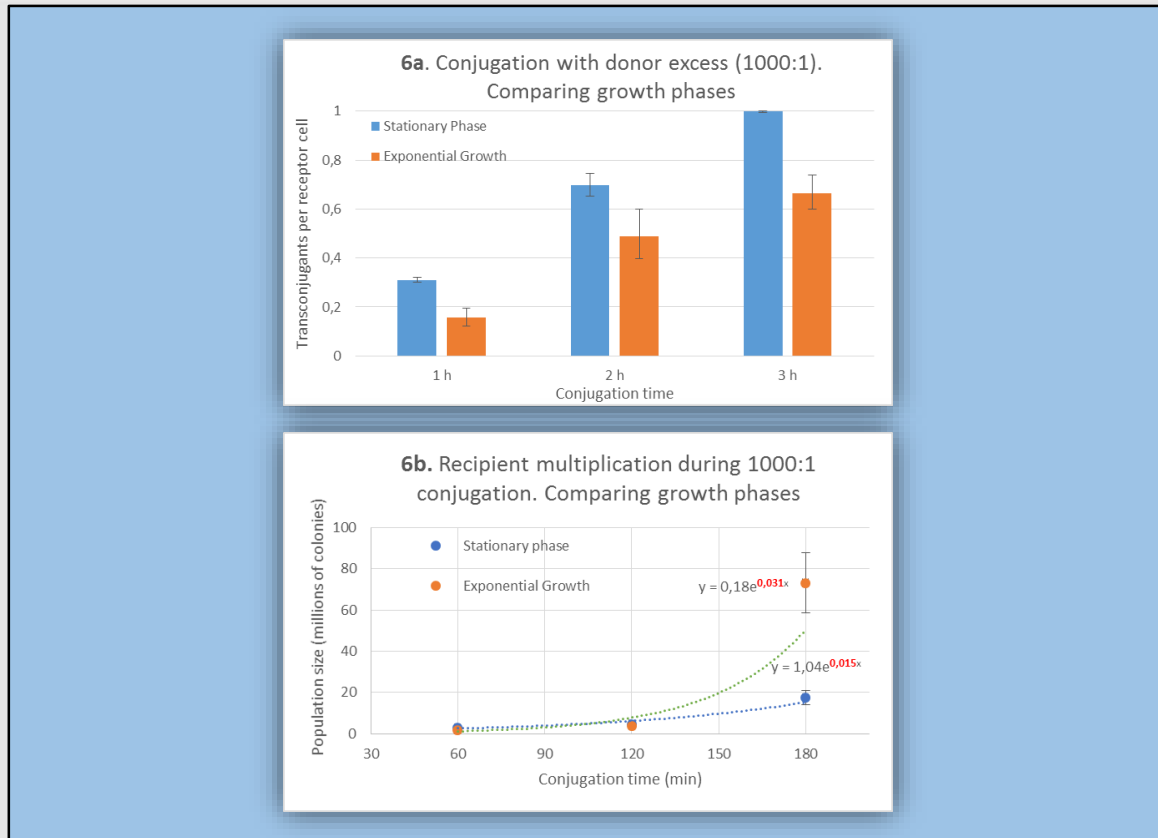


*Fig. 4. Increasing amounts of donor cells were mixed with a fixed amount of recipient cells. Great abundance on donor cells do not show to improve the number of transconjugants generated at 1 hour.*

The latter experiment was repeated, using this time 1000:1 proportions of Donor:Recipient, stopping conjugations at 1 hour, 2h and 3h. If there was a conjugation-inhibited subpopulation this experiment would ultimately show it up. Number of transconjugants was resolved by replica-plate method (see Materials & Methods). At 3 hours every recipient had become transconjugant:

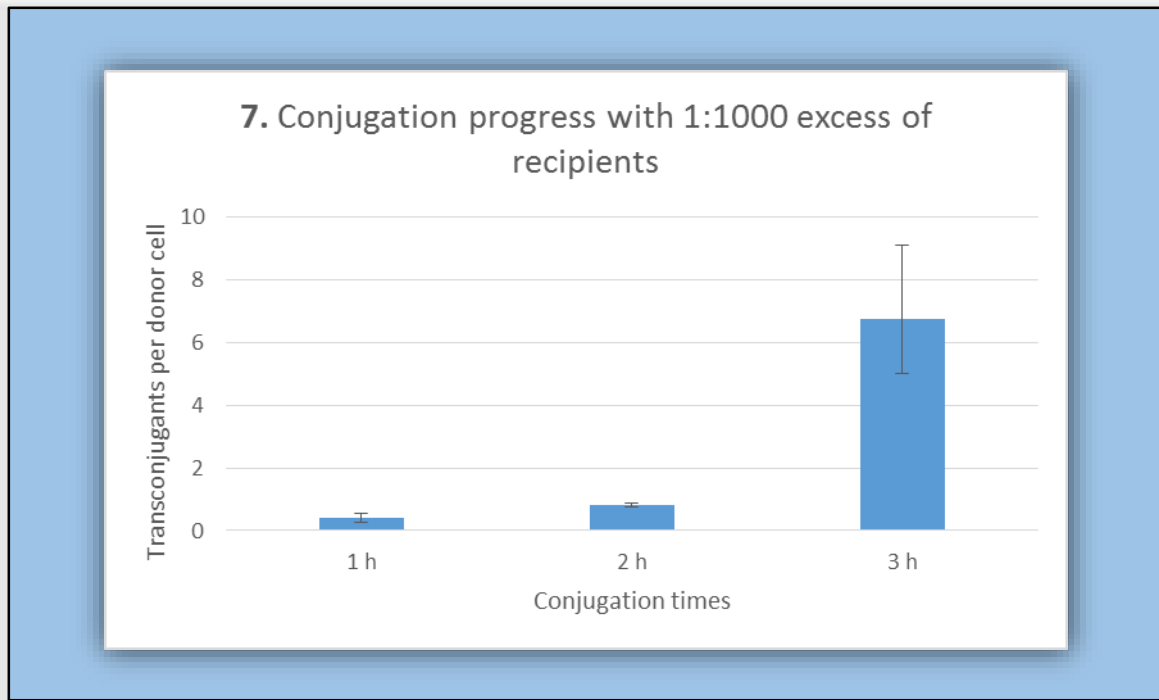


The same experiment, using 1000:1 proportions was made, but this time bacterial cultures in exponential growth were used besides the usual stationary-phase cultures in order to show growth effects (**Fig. 6a**). Recipient bacteria in exponential growth showed double-speed growth (see **Fig. 6b**) and also decreased conjugation efficiency: 100% of plasmid acquisition in the recipient population was not accomplished at 3 hours. As discussed later, it may be due to density effects.



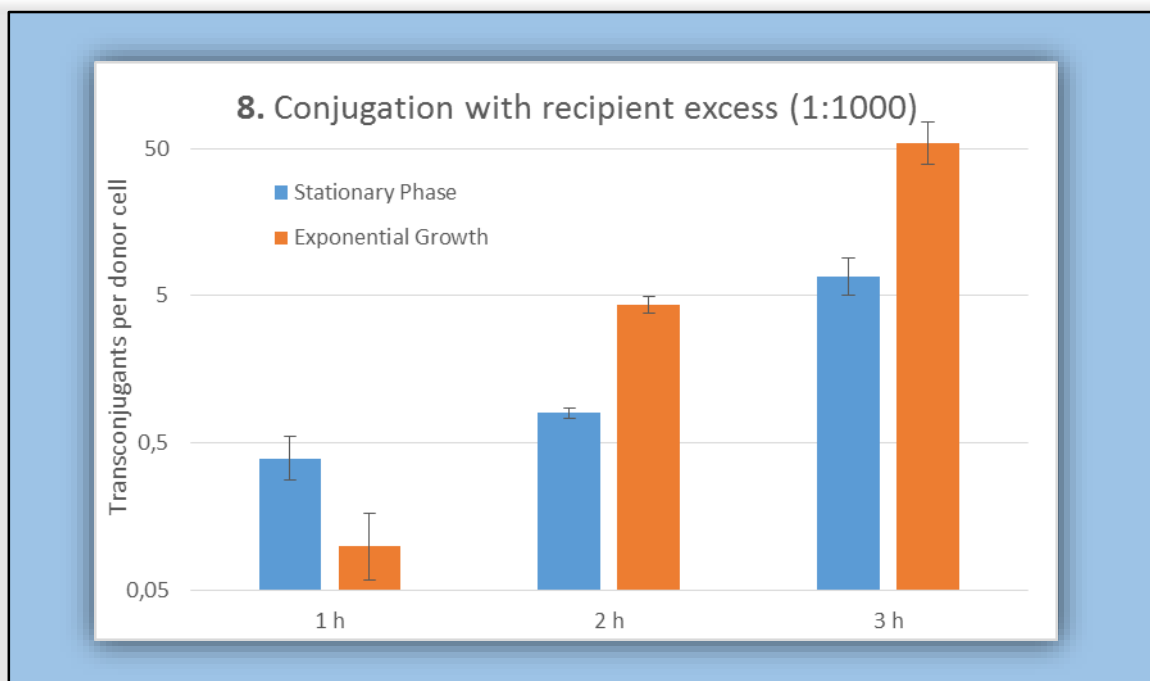
**Fig. 6. Conjugation efficiencies at 1 hour, 2 hours and 3 hours. Comparison between exponential growth phase conjugation and stationary phase can be seen in Fig 6a; growth rates of recipient cells are underlined in red in Fig 6b for both stationary and exponential phase cultures.**

In order to know how much transconjugants can a single donor create, a tiny amount of R388<sup>+</sup> bacteria was inoculated in a dense population of bacteria lacking the plasmid (1:1000 ratio Donor:Recipient). As precedent results pointed before, at 1 hour the numbers of transconjugants had not reached the number of donors (see **Fig.7**). However, when the mating continues for 2 and 3 hours, the transconjugant population shows a substantial burst.



*Fig. 7 Number of transconjugants at 1, 2 and 3 hours in a conjugation experiment with almost unlimited recipients per donor cell. Exponential growth of the number in the number of transconjugants shows the ability of R388 to spread in a crowded recipient population.*

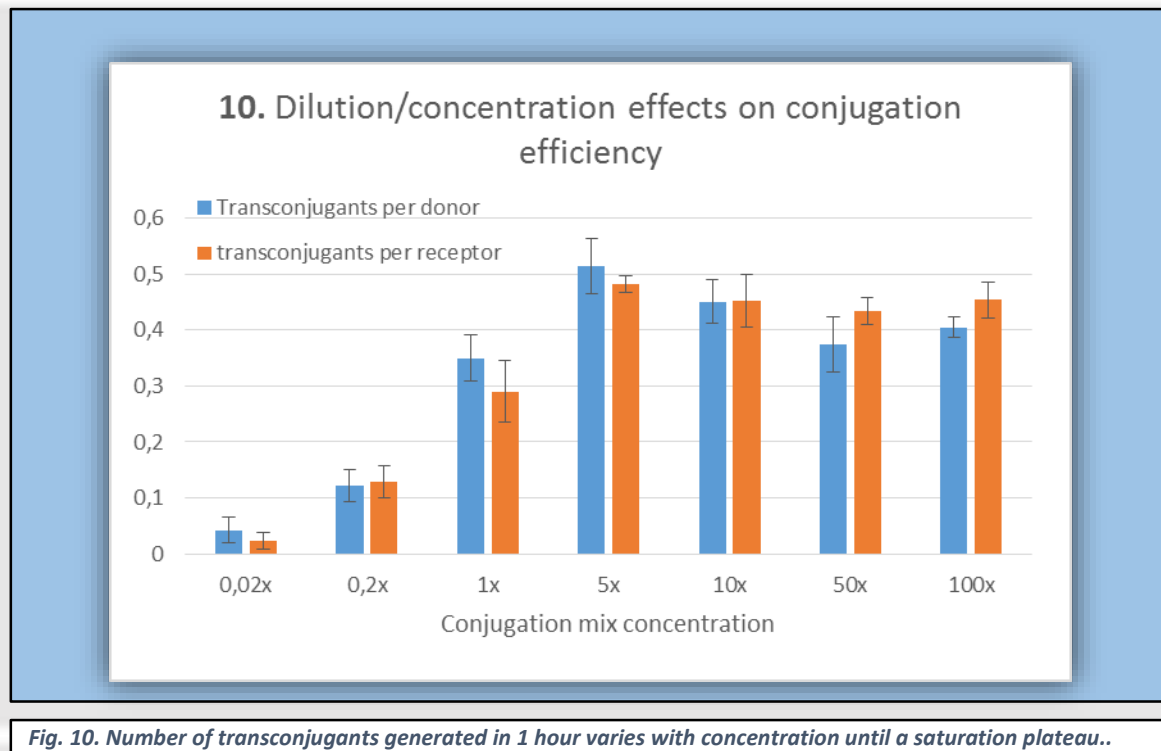
The 1:1000 experiment displayed in **Fig.7** was repeated comparing the former results with those achieved using bacterial cultures at exponential growth phase. Conjugation efficiency is shorter in exponential experiments due to density issues. Transconjugant population burst was even bigger in this case (see Fig. 8):



*Fig. 8. Comparison between conjugation at stationary phase and exponential phase was made. Number of transconjugants per donor cell in this graph is represented in logarithmic scale. Even bigger growth burst of transconjugants may be observed for the exponential growth phase.*

### Density vs. Frequency dependence

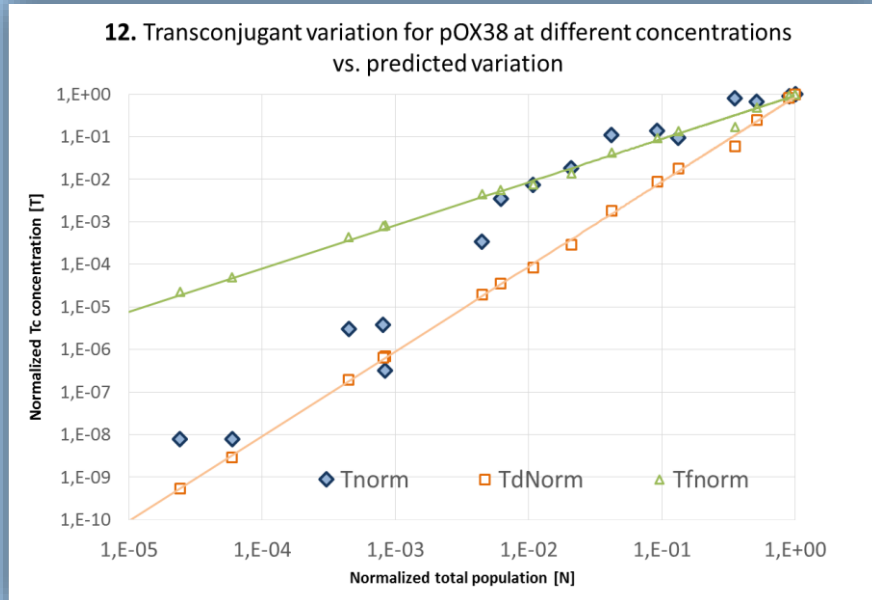
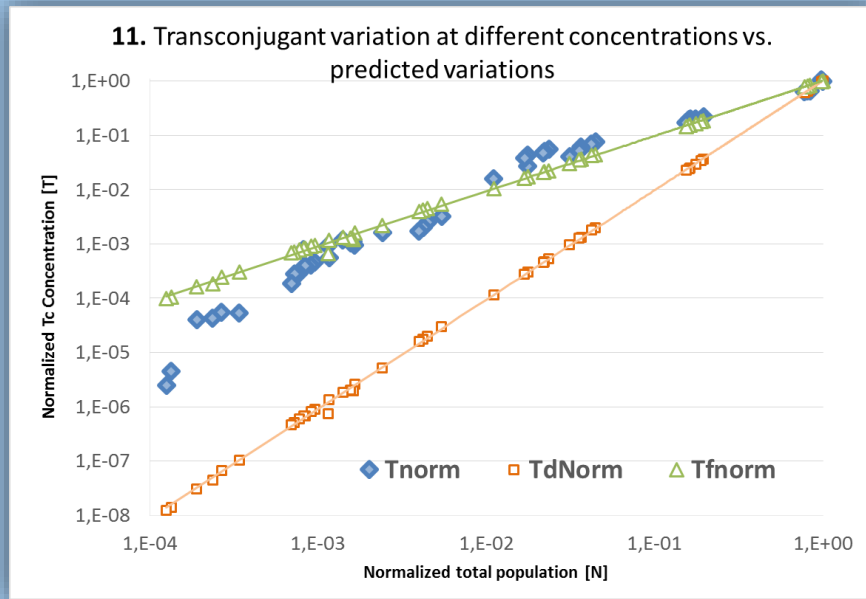
Density effects on the conjugation efficiency were tested by concentrating or diluting the conjugation mix before being plated. 1:1 Donor: Receptor proportions were used. Due to experiment design issues it was not possible to extend far away the ranges of concentrations and dilution. Conjugation efficiency – measured as the number of transconjugants produced per each donor or recipient cell- increases with density until it gets to a plateau All conjugation experiments in stationary phase described before in this work were performed at a concentration equivalent to the 10x indicated here.



Density-Dependent Transmission (DDT) and Frequency-dependent Transmission (FDT) models (see Materials & Methods) were applied to predict how the concentration of transconjugants should vary when varying the population density. DDT predicted a quadratic increase of normalized  $[T_c]$  when increasing normalized  $[N]$ , whereas FDT model predicted a linear increase.

Applying this predictions to the dilution mating experiments shows a FDT-like fashion for almost all the range of dilutions (see Figs 11 and 12). The dilution-concentration experiments were developed also in liquid (LB) medium. For these experiments bacterial strains used were the same, however, not R388 but pOX38 was used instead (see Materials & Methods). Conjugations were performed for 1 hour at 37°C.





*Fig. 11 and 12. Tc normalized concentration vs normalized total population. Axis are expressed in logs for apprehensibility puprposes. In green the predicted transconjugant concentrations normalized for a Frequency-Dependent Transmission system, showing a linear (slope=1 in log scale) dependence with N. In blue the predicted transconjugant concentrations normalized for a Density-Dependent Transmission system, showing a quadratic variation with N (slope 2 in log scale). The conjugation results (in blue) show a FDT-like fashion.*

Normalized concentrations of transconjugants are represented vs. the normalized concentration of total population (see **Fig.12**). A FDT-like fashion can be observed for the conjugation of this plasmid in liquid medium, although it decays at high dilutions due to spatial issues discussed later.

## PART II. Modulation

As conjugation showed to be auto-limited by donor cells and not to be limited by availability of recipients, the next step was if changing the stoichiometry of the relaxosome and its relative proportion and its relative proportion with the pilus could eliminate partially or totally those limitations.

### Conjugation and complementation setup

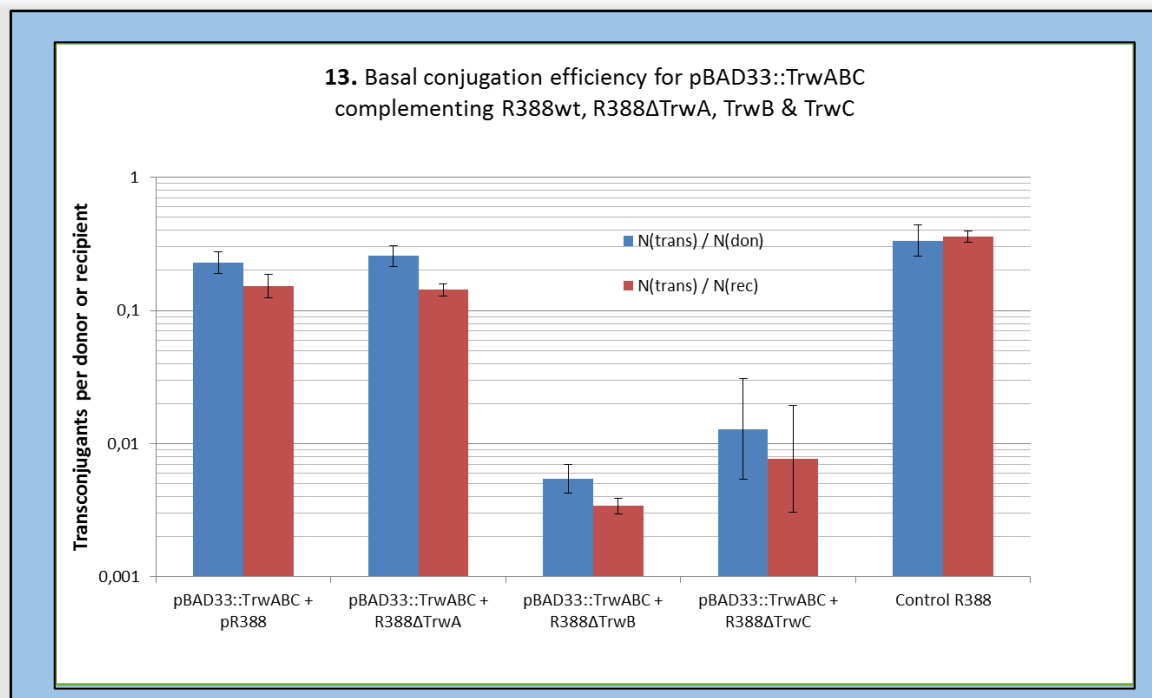
At first, conjugation with proportions 1:1 of donors and recipients were performed with plasmids lacking one of the relaxosome (*trwA*, *TrwB*, and *TrwC*) genes. Conjugation was only achieved with the *R388ΔTrwA*, and number of transconjugants was around 1000 times less than conjugations with the *wtR388*. Conjugation with *R388ΔTrwB* or *R388ΔTrwC*, if existent, was under one transconjugant generated per ten million donor or recipient cells.

Complementations were performed with *pBAD33* clones carrying *TrwA*, *B* and *C* as described in Materials & Methods. In all cases, conjugation was restored to wild-type levels.

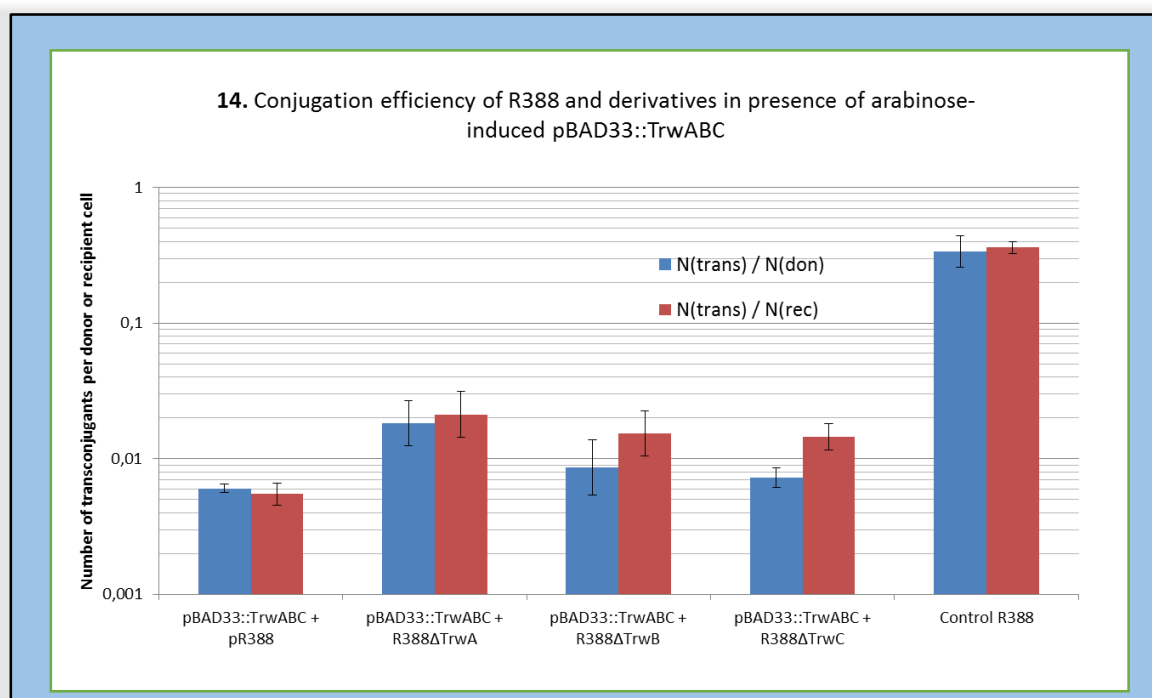
### Whole relaxosome modulation

Next round of experiments was aimed to modulate the relaxosome as a whole. A *pBAD33::TrwABC* plasmid was designed and transformed into *wt-R388+* donor cells and into all the *R388ΔTrwA*, *B* or *C* carriers. *pBAD33* plasmid allows to modulate expression, increasing it with arabinose and decreasing it with glucose. Basal complementation (**Fig. 13**) shows that only small (basal *pBAD* expression) amounts of *TrwA* are needed to achieve wild-type conjugation:

Experiments **repressing** *pBAD33::TrwABC* with glucose resulted in conjugation efficiencies similar to those obtained when no plasmid construct was involved on *R388* and the three deleterious derivatives: undetectable for the *R388ΔTrwB* and *R388ΔTrwC*,  $10^3$ - $10^4$  times shorter for *R388ΔTrwA*.



**Fig. 13. Conjugation efficiencies as number of transconjugants per donor (blue) or per recipient (red) cell are shown. Basal addition of pBAD33::TrwABC increases conjugation efficiencies in the three deleterious mutants.**



**Fig. 14. Conjugation efficiencies as number of transconjugants per donor (blue) or per recipient (red) cell are shown. Addition of pBAD33::TrwABC induced with arabinose 0.025% decreases conjugation efficiency, nonetheless, when no wt-TrwA is present (second column) conjugation slightly increases.**

Experiments **over-expressing** the whole operon TrwABC (see **Fig.14**) were performed adding 0.0025% vol. of arabinose. As long as an important decrease in conjugation efficiency was immediately observed -and the aim of these experiments was to improve that efficiency- further arabinose concentrations were not tested.

# DISCUSSION

## Conjugation setup

Bacteria carrying plasmid R388 has been reported to have a burden that increases their generation time slightly with respect to the same strain lacking the plasmid. (30). This might bother the characterization of conjugation measurements. However, both recipient and donor cells were used in stationary phase and conjugations performed for 1 hour, so that demographic issues could be neglected.

Number of transconjugants per recipient cell falls to almost 50% when recipient and donor cells are cultivated for more than half a day. This could be related to reported loss of superinfection immunity in late stationary phase (J. Lederberg, et al. "Sex compatibility in *Escherichia coli*" *Genetics*, 37 (1952), pp. 720–730), which eventually could suppose an impaired selectivity in the election of recipients, or it might also be attributed to toxic effects due to secondary metabolism and by-products present in this growth phase. Further studies would have to be developed in order to solve this.

Although big noise is associated to conjugation efficiency measurements, almost no one of these first experiments reached to fulfill the recipient population with R388 plasmid. One of the indirect objectives of this study was to achieve the necessary conditions to make this possible in at least 1 hour.

## Conjugation kinetics

As results seem to show, conjugation takes some time (around 60 minutes) to proceed since the mating contact is carried out. But also, as almost every cellular process it is bound to stochastic dynamics, making the synchronization in the lag only visible for the first row of conjugation events, after that, a continuous increase of transconjugant population can be observed as secondary events blend with ulterior conjugations from the original donors.

As long as replication could distort the results of this experiment, only stationary phase cultures were used. No growth can be appreciated in recipient or donor populations in conjugation mix nor in its own (control).

### **Conjugation limits**

Putting a plenty of donors for a few recipients was expected to increase the number of transconjugants as they would be able to get in touch more probably with donor cells. This expected behavior was not observed at 1 hour, keeping limited the proportion Tc/Rec at about 0.5 every experiment. This raised the troubling question of whether there was some sub-population of recipients somehow unable to receive the plasmid, or even actively rejecting the conjugation.

Answering this question was possible by putting a huge excess of donors (1000:1), so as every recipient was, for sure, surrounded by donors. In order to assure time for conjugation, 3 hours were elapsed since mating began. At 3 hours, every recipient had become transconjugant. For this experiment, selection of transconjugants (see Materials & Methods) was carried out with replica method, as inherent noise linked to usual protocol, (that involves measurements of populations bound to stochastic phenomena) would keep the uncertainty about inhibited subpopulations.

It deserves to note that the proportion of transconjugants in one hour at this huge excess of donors, was exactly the same that in 1:1 proportion, pointing this result to a limitation of the donor and independence on the proportion of Donor:Receptors, as described for conjugation of plasmid R1 in (29).

The experiment was completed by using cultures with cells in exponential growth. The results show decreased rates of conjugation due to: 1) design of the experiment was focused on maintaining the exponential growth, so diluted cultures and highly diluted mating mixes were used, what hampers mating possibilities, as shown later; 2) recipient cells kept growing at the same pace while transconjugants suffered from the big burden of receiving the plasmid, and its associated overshoot, (Negative Feedback and Transcriptional Overshooting in a Regulatory Network for Horizontal Gene Transfer) stopping replication for a while.

## **Conjugation in almost wholly susceptible medium**

The inverse experiment was developed: by putting a minimal amount of donors among a vast population of recipients (proportion 1: 1000) the definition of  $R_0$  (Anderson R, May R (1992) *Infectious Diseases of Humans*. Oxford: Oxford University Press: “the average number of secondary infections produced when one infected individual is introduced into a host population where everyone is susceptible”) would be nearly accomplished. However, transconjugants generated by the original donors, and transconjugants generated by early transconjugants are impossible to differentiate with our experiment design.

The results of this experiment show that plasmid spreading in the recipient population follows a geometrical increase, as noted before in (30). This geometrical increase outnumbers in about 2 hours the number of original donors. However,

As discussed before, it is not easy to discriminate which of those newly formed transconjugants are due to “secondary transmissions” or come from original donors.

In order to calculate  $R_0$  it would be necessary to have a method to avoid conjugations from the recently formed transconjugants. This is explained in more detail later on.

Transconjugant to donor proportion grows even faster in exponential growth phase experiments, what is particularly striking given the greater dilution conditions, the superior growth rate of donor cells over the new transconjugants, and the low rate of transconjugant formation at 1 hour.

## **Secondary conjugation**

Preliminary results showed that secondary conjugation plays an important role in plasmid spreading, as long as it allows a geometrical expansion dynamics. With no secondary conjugation it would be easier to calculate the parameters of conjugation. The problem is not to make a model plasmid that only conjugates once, as long as it is the essential feature of non-conjugative, mobilizable plasmids. The problem is to build a plasmid model with similar rates of conjugation to the wild-type R388, but unable to go further the first recipient.

Three models were used, two of them based on separated plasmids with the complete R388 conjugation machinery, but with only one plasmid in each case carrying the *oriT*. The other model used the “fertility inhibition” described for some plasmids (R6K in this experiment) over R388.

In the first two cases conjugation efficiency decreased significantly at 1 hour (around 100 times



smaller) and made it difficult to compare the results with those from the wild type plasmid. The last experiment seems to be a better candidate in order to study direct conjugation alone, but further studies have to be performed.

### **Density and Frequency Dependent Transmission**

Experiments on diluted and concentrated mixes of conjugation showed increased formation of transconjugants when concentrating the cultures, but in mixes more concentrated than those obtained with the protocol we use in this work, proportion of transconjugants does not grow, pointing to some limiting factor.

Two probable explanations might address this problem: the first one, a geometrical barrier is imposed, the plate is crowded and increasing the concentration is no longer feasible as long as no more recipients can be put in contact with the donor. The second one: a limit on the number of possible conjugations performed in this period of time by the donor exists.

Note that these two explanations are not mutually exclusive. But in this case, where using 1.6 cm diameter wells of LB-agar, with an estimated surface of about  $10^9 \mu\text{m}^2$ , there are around  $10^8$  bacteria (in the 10x concentration), with an estimated surface of  $1 \mu\text{m}^2$ . That means that saturation of the conjugation process would not have to show up at least until 100x concentrations were reached.

This pointed to a Frequency-Dependent transmission with the maximum efficiency at 0.5 transconjugants per donor or receptor cell in one hour, with a loss of efficiency as dilution increases, because mating pairs become rare and difficult to find in solid state.

Contrasting these results with the two available models of transmission (revised in Materials & Methods), R388 showed a Frequency dependence that only disappears, as explained in the paragraph before, when very low amounts of cells are present in the mating plate and contact becomes almost impossible in one hour.

Applying the same method to a plasmid able to conjugate in liquid, pOX38, conjugation showed also frequency dependence for this plasmid. Loss of this dependence at not-so-diluted levels can be attributable to the tridimensional features of conjugation in liquid: with more or less the same amounts of cells, but distributed along a 1 cm. height column, is more difficult to get into contact. In raw numbers it would make around  $10^{13} \mu\text{m}^3$  for  $10^8$ - $10^9$  bacteria occupying less than  $1 \mu\text{m}^3$  each. Letting apart diffusion and agitation issues, it has to be a difficult deal for these bacteria.

## Modulation of relaxosome operon

The operon TrwABC is responsible for initiation of the conjugation process. It is known as the *dtr* (DNA transfer replication) and it is 5200 base pairs long. The three genes are repressed by the product of the first one, TrwA, which is also the *oriT*-binding protein. This auto-regulatory mechanism, known as Negative Feedback Loop (NFL) generates the transcriptional overshooting described in (30) when R388 enters a recipient.

The main objective of this set of experiments was to boost this overshooting for reaching increased conjugation rates.

The first result, complementation of individual genes, showed that *trwA* has an important role in conjugation, but it is not essential for the process.

The following experiment has three parts:

- 1) Basal complementation. This experiment showed partially recovered conjugation for R388 $\Delta$ TrwB and R388 $\Delta$ TrwC and almost totally recovered conjugation efficiency for the R388 $\Delta$ TrwA.
- 2) Repression with glucose. This experiment showed similar results to those obtained with no pBAD33 construct. Reduced conjugation for R388 $\Delta$ TrwA, inexistent for R388 $\Delta$ TrwB and R388 $\Delta$ TrwC.
- 3) Induction with arabinose. An excess of the operon TrwABC did not improved conjugation efficiency, but impaired it in all mutants and the wild type donor.

From these results it appears that TrwB and TrwC are strictly necessary for the conjugation to start. TrwA is also important, but not essential.

It remains unclear if basal production of pBAD33::TrwABC was not able to recover the conjugation efficiency when combined with R388 $\Delta$ TrwB and R388 $\Delta$ TrwC because it was not able to recover the normal, stoichiometric quantities needed, or if the impaired conjugation efficiency is due to an excess of the two complementary proteins. This question is fueled with the results of operon induction, which shows that excess of any of the three proteins hampers conjugation.

## CONCLUSSIONS and FUTURE PROSPECTS

Several mating experiments have been performed bringing a conceptual framework about the conjugation of R388 in *E. coli*:

Conjugation is limited by both donors and recipients. That limit is measurable and does not depend on density for a broad range of population densities.

We can conclude that conjugation follows Frequency-Dependent Dynamics; this is fundamental in order to develop population models on dissemination of antibiotic resistance genes.

Further studies have to be carried out to determine more precisely conjugation rates with no secondary conjugation, to study conjugation rates in vivo and finally, demographic and metabolic effects have to be measured to be included in our model.

The main objective of this work was to develop tools to quantify the process of conjugation in order to build a mathematical model of plasmid spreading in real environments.

Future experiments to be developed in this respect are, in the short term:

- Tuning of primary and secondary conjugation, mobilization of other plasmids, and duplication measurements.
- Extension of conjugation measurements on different plasmids to generalize the results of FDT in R388 to the conjugative plasmids plethora.

In the mid-term, all these measurements, should allow to:

- Generate a fine-tuned mathematical model of plasmid spreading in solid surfaces.
- Adapt it to get ready microfluidic mating experiments and in vivo (mammalian gut) mating experiments; an approach already tested for other plasmids but not finely quantified.
- Extend this model to real systems with spatially structured populations such as biofilms in bioreactors and conjugation in gut mucous membranes.

In the long term, the objectives will be:

- To perform directed evolution experiments that generate R388 derivatives with improved (or impaired, if it should be) infectivity.
- To build computational systems that may use fine-tuned conjugation as communication system.
- To engineer therapeutic and ecological resistance genes blockers, using conjugation

as method to spread and selectively attack the multi-resistant strains.

Finally, it would be interesting to deepen the study on modulating the conjugation components, although the first approach has not been very promising. This negative results may be due to the difficulty to alter a very intricate regulatory network that evolved for optimal phenotype in its real biological conditions.

These “real” conditions are powerfully determined by the trade-off between internal fitness and host fitness: plasmids must evolve in such a way to ensure their vertical transmission (reducing the physiological burden for the host), but also enhancing horizontal transmission in order to spread in the population.

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# FREQUENCY-DEPENDENT TRANSMISSION IN PLASMID CONJUGATION



Jorge Rodriguez Grande\*, Fernando de la Cruz Calahorra\*  
\*Instituto de Biomedicina y Biotecnología de Cantabria (IBBTec) UC-CSIC Av Albert Einstein 22 39011 Santander



## INTRODUCTION

Horizontal Gene Transfer (HGT) of plasmids and Integrative and Conjugative Elements (ICEs) mediated by conjugation is one of the most important mechanisms allowing the spreading of multi-drug resistances [1]. In order to plan effective measurements to stop the dissemination of antibiotic resistances, we need to understand the population dynamics of plasmid transfer. Conjugation is a biological process in which donor bacteria transfer a plasmid to recipient bacteria mediated by transfer proteins encoded in the conjugative element [2].

Previous works on conjugation rates on solid surfaces have provided information on primary transfer events [3]. Nevertheless, more data on transfer kinetics is needed to model mathematically the conjugation process, a basic requirement to predict plasmid dissemination in bacterial populations.

The common accepted model, known as “density-dependent model”, assumes that spreading of plasmids has a transfer rate limited only by direct contact between a donor (infected) and a recipient (susceptible) cell, following a dynamic similar to that of flu epidemics [4].

In this work plasmids R388 and F were used as infecting agents to test the rate of plasmid transfer regarding the availability of susceptible *E. coli* recipients. Contrary to the original model, our results suggest that plasmid spreading does not follow a density-dependent dynamics, but a frequency-dependent one. This has important implications in the kinetics of plasmid dissemination and the mechanism of plasmid transfer.

## MATERIALS AND METHODS

**Mating experiments.** They were carried out using the *E. coli* strain Bw27783 (Nx<sup>R</sup>) as a donor strain of the conjugative plasmid (either R388 (Tp<sup>R</sup>), or pOX38 (a Km<sup>R</sup> derivative of the plasmid F)) and Bw27783 (Rif<sup>R</sup>) as a recipient strain.

Different ratios of donor and recipient strains were used, estimated by OD<sub>600</sub> measurements. Donor and recipients were mixed and plated onto LB-agar surfaces in a 24-well plate, at 37°C, for periods ranging from 1-3h.

After mating, the cells were resuspended in PBS to avoid subsequent growth and dilutions were plated in appropriate antibiotics. Conjugation frequency was calculated as the proportion of transconjugants per donor or recipient cell.

Some mating experiments were carried out with serial dilutions from the original cultures of donor and recipient cells.

Charts indicate the average and confidence interval (significance level: 0.05) for n=9 replicates. They were calculated taking into account that rates of conjugation follow a log-Normal Distribution.

### Mathematical model.

In epidemiology, the so-called “Force of Infection” (FOI or  $\lambda$ ) is used to measure infectivity of a pathogen.

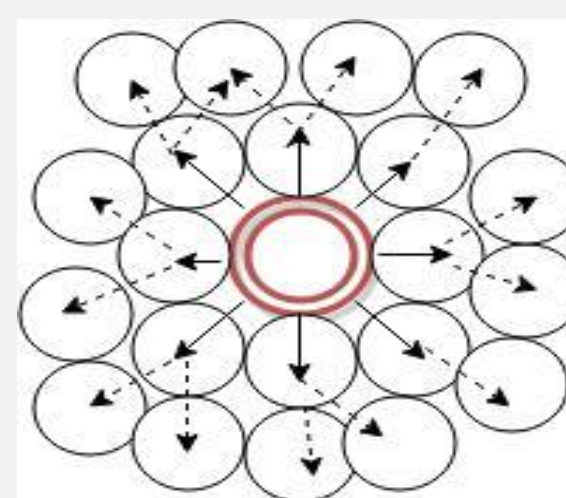
- $r$  : rate of encounter per cell
- $P_i$ : Probability of encounter being between donor and receptor.
- $P_t$ : Probability of that encounter resulting in transmission.

$$\lambda = P_i \cdot P_t \cdot r$$

Growth on “infected” individuals will be the product of FOI and the number of susceptible individuals:

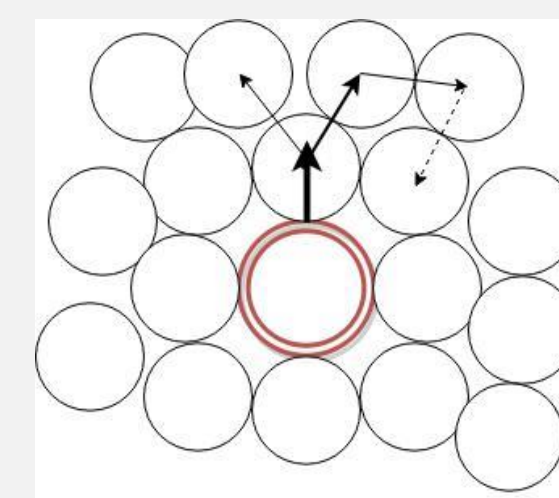
$$\frac{dT}{dt} = \lambda \cdot N_{\text{rec}}$$

The Density-Dependent Transmission (DDT) model relies on the idea of transmission being only a matter of availability of receptors. On the other hand, the Frequency-Dependent Transmission (FDT) model considers the phenomenon as a saturable transmission from the donor to the receptor:



Density-Dependent

$$\lambda = \left( c \cdot \frac{N}{V} \right) \cdot \left( \frac{D+T}{N} \right) \cdot v$$



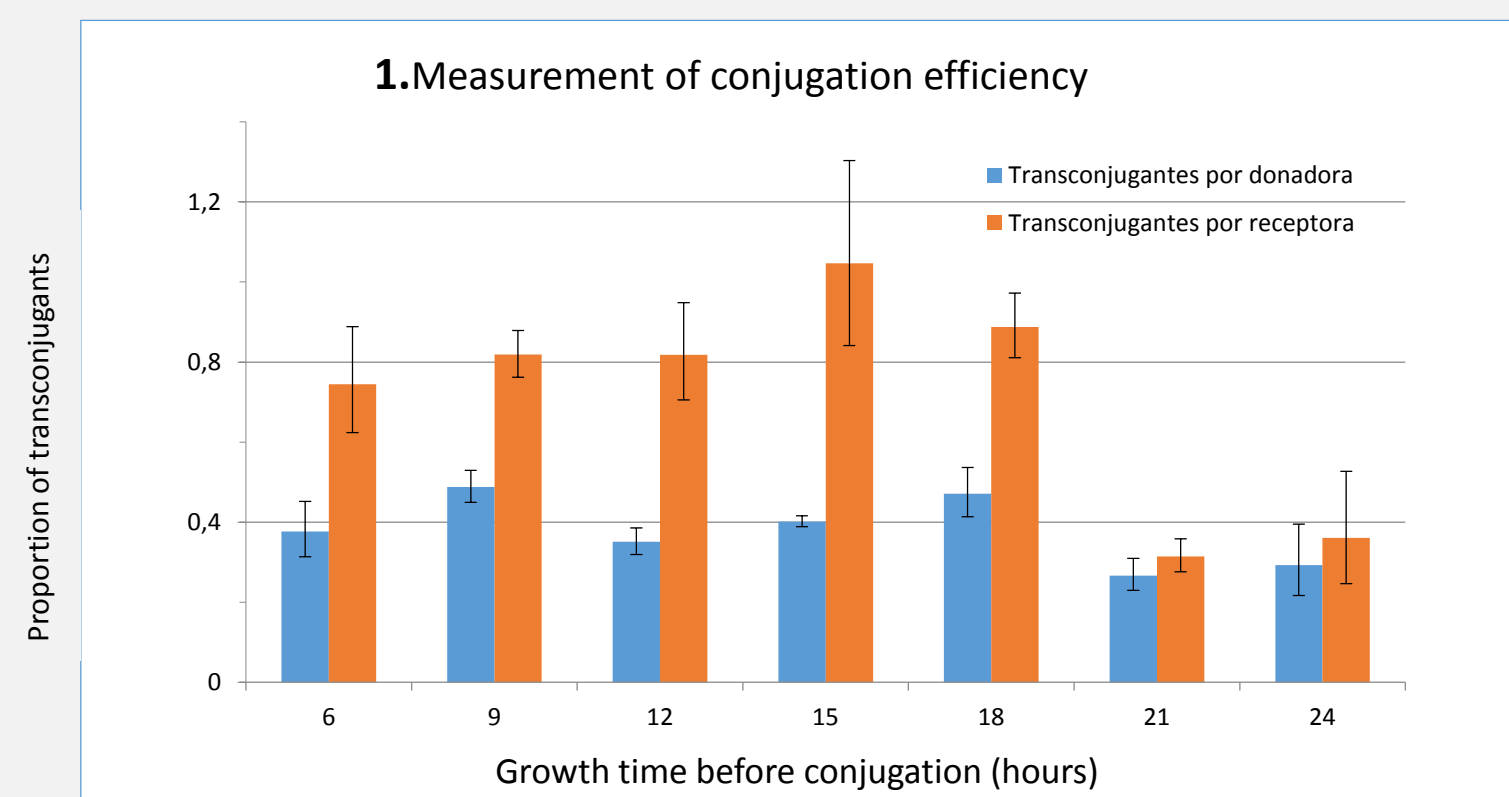
Frequency-Dependent

$$\lambda = c \cdot \left( \frac{D+T}{N} \right) \cdot v$$

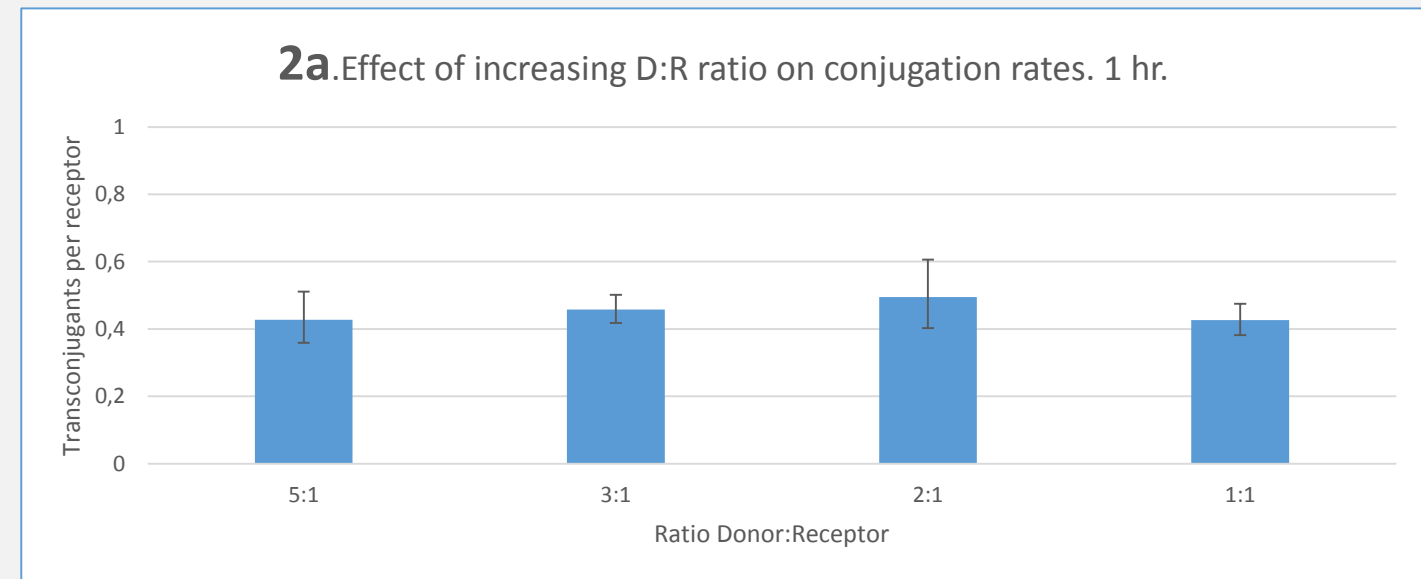
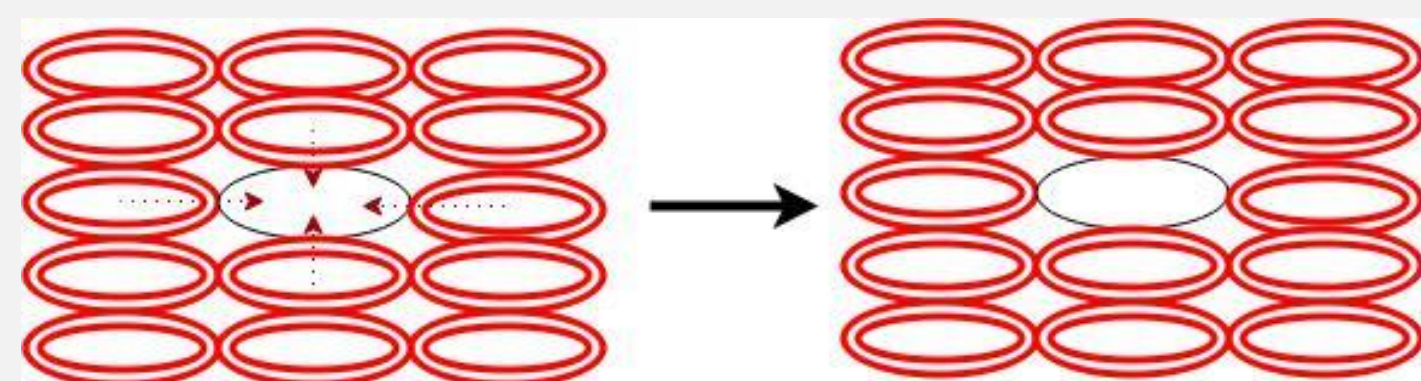
Serial dilution mating experiments in both liquid and solid media, were used to construct normalized concentration diagrams of population in order to test which of these two mechanisms is the one that R388 and F follows.

## RESULTS

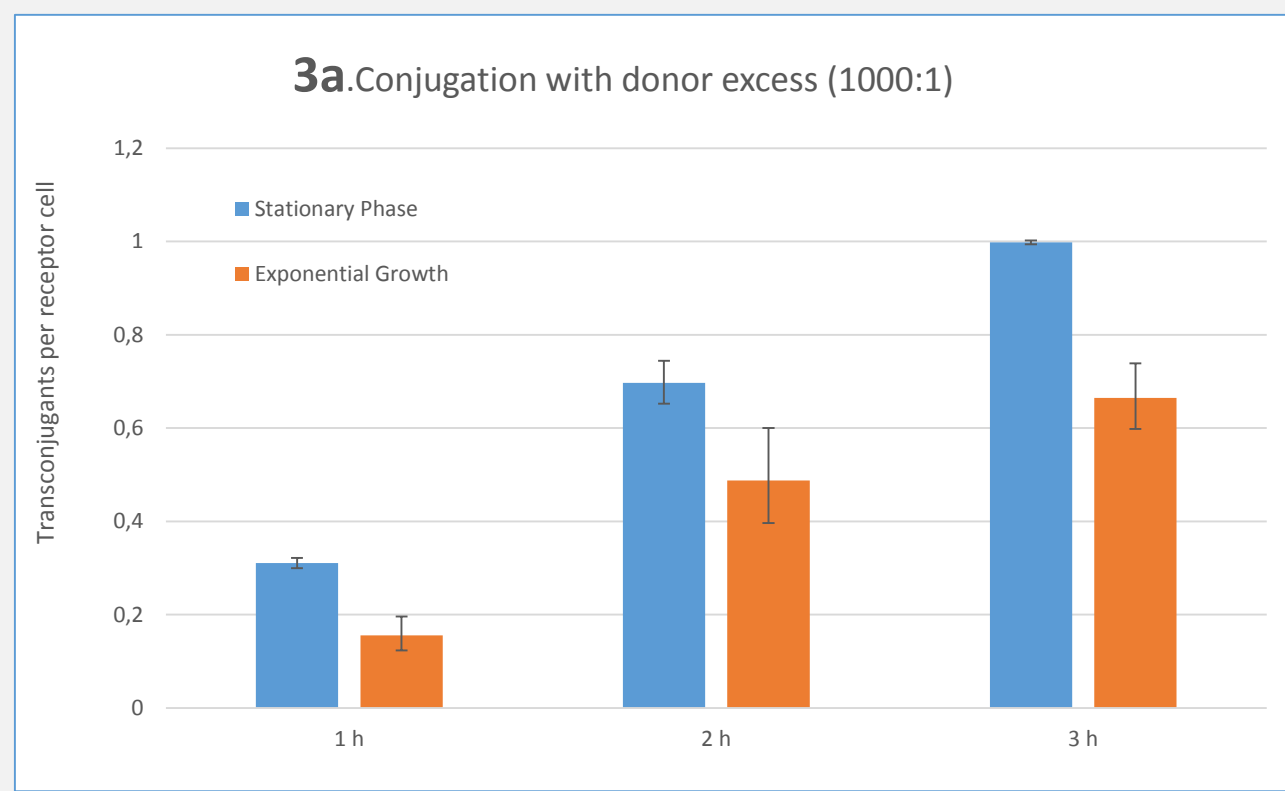
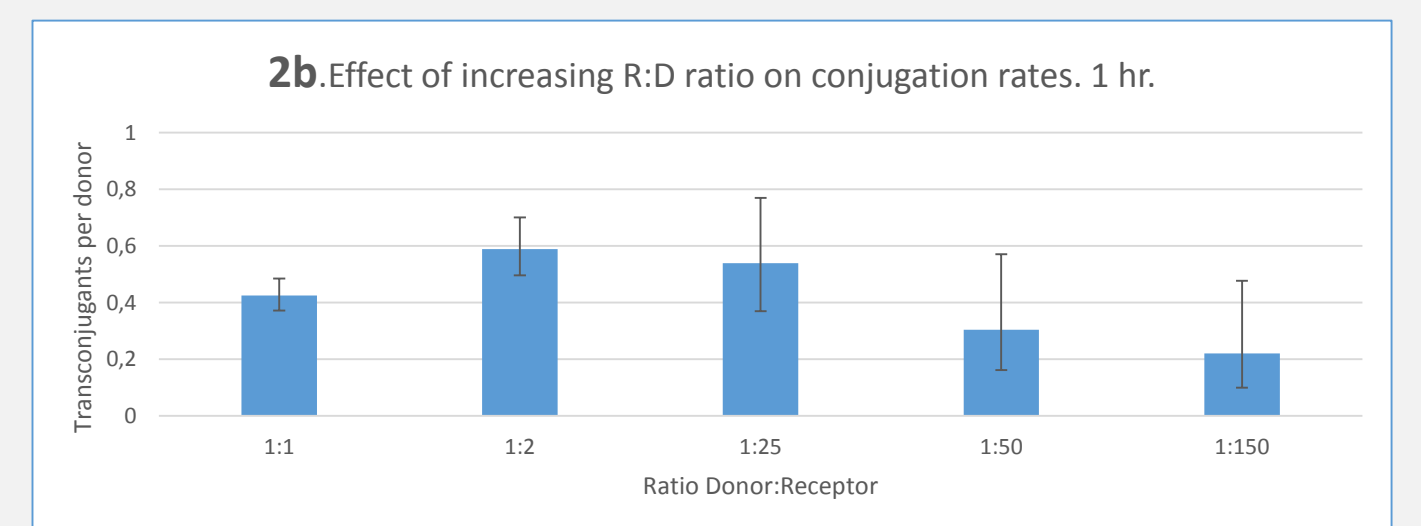
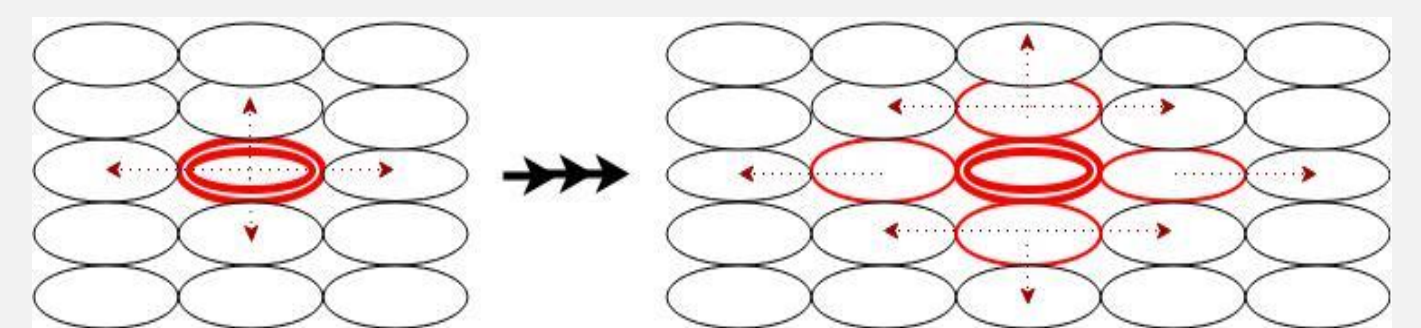
**1.**First rounds of experiments were developed with a 50% proportion of donors and receptors. Initial measurements showed noisy, but stable, results except for aged cultures :



**2a.** Is there any limit imposed by recipients that could limit conjugation temporally?



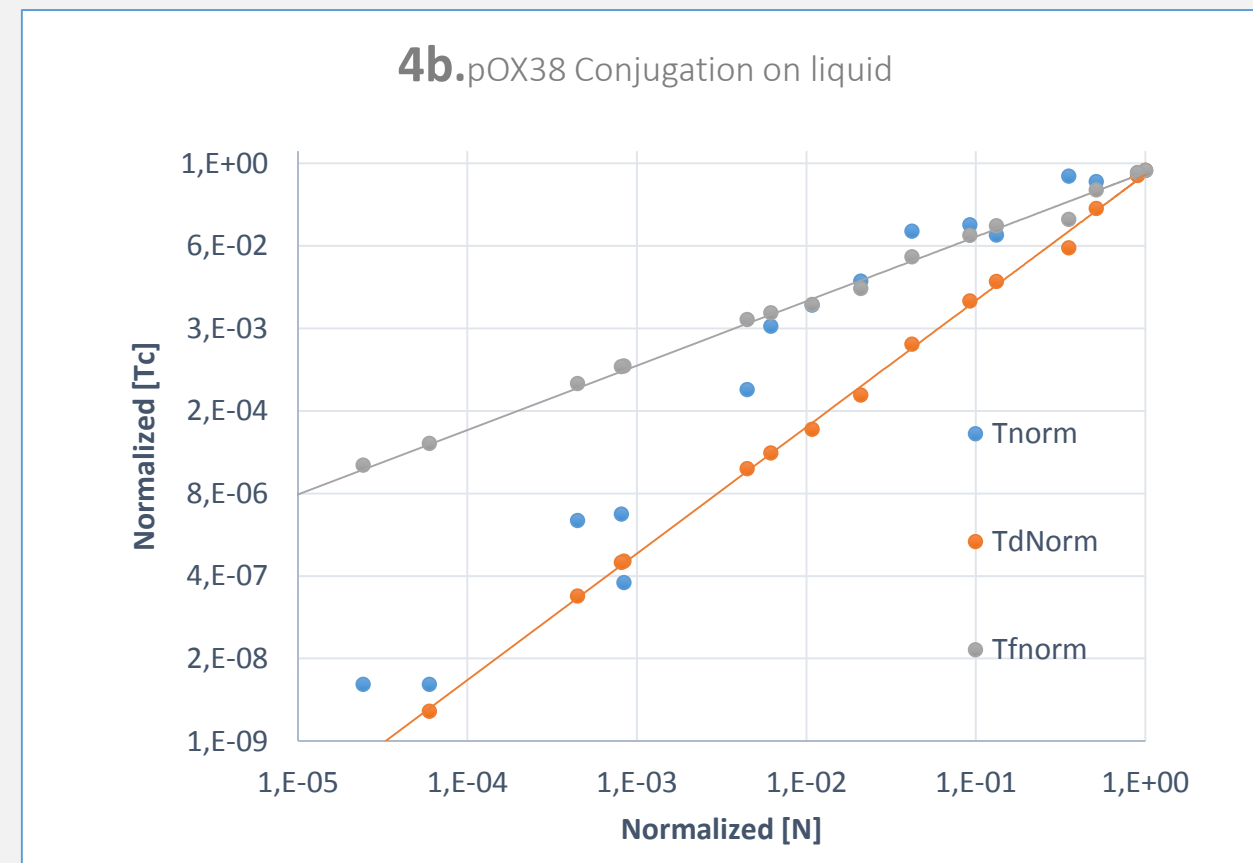
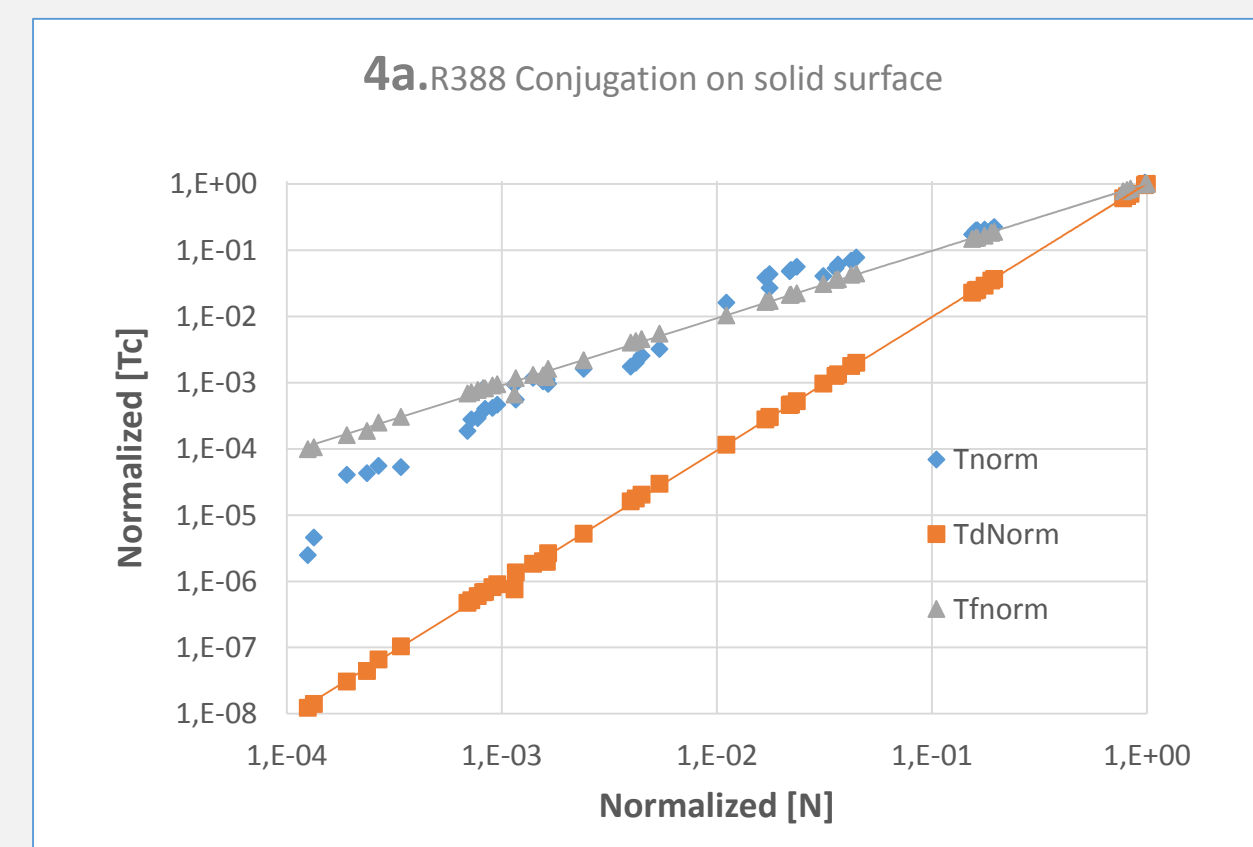
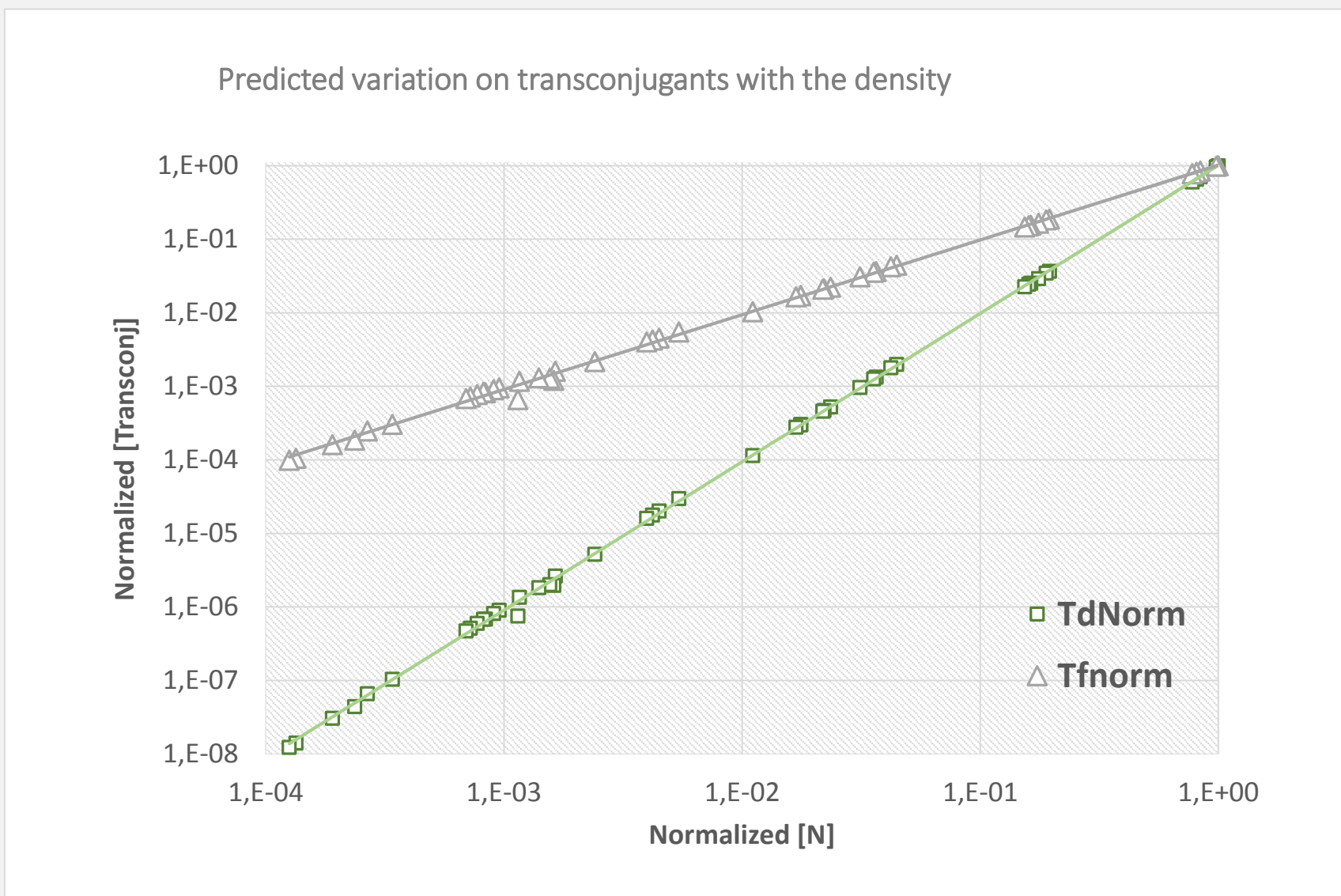
**2b.** Is there a limit to the number of conjugations a donor can carry out in one generation time?



**3a.-** Longer mating times resulted in plasmid delivery to the 100% of recipient cells when donors outnumbered recipients by 1000 to 1. This suggests that **all recipient cells are susceptible to accept conjugation events**, the limit would not be in the number of donors or recipients but instead, it would be in the mating time and the time required for the cell to reach the optimal metabolic state. When donor and recipient populations were mated in exponential phase, conjugation rates decreased. Further studies on physiological state and demographic effects have to be done.

**3b.-** Longer mating times with great excess of recipients (1:1000) shows increased transconjugant formation speed, probably due to secondary conjugation events with transconjugants acting as donor cells after a recovery time.

**4.-** Normalized [Tc] follows a Frequency-Dependent-Transmission fashion in almost all the range in solid (Fig.4a); transition to a density-dependent regime emerges at very low concentrations as recipient and donor cells become scarce. More pronounced deviations in the liquid experiment (Fig.4b) are attributable to 3D diffusion.



## CONCLUSIONS:

- Conjugation is limited by both donors and recipients. That limit is measurable and does not depend on density for a broad range of population densities.
- We can conclude that conjugation follows Frequency-Dependent Dynamics; this is fundamental in order to develop population models on dissemination of antibiotic resistance genes.
- Further studies have to be carried out to determine more precisely conjugation rates with no secondary conjugation, to study conjugation rates *in vivo* and finally, demographic and metabolic effects have to be measured to be included in our model.

## FUTURE PROSPECTS

- In the short term this study paves the way to: a) build mathematical models of plasmid dissemination and b) to test directed evolution experiments to improve conjugation rates.
- In a longer term we would like to create molecular tools to stop plasmid dissemination and generate bacterial computers based on plasmid conjugation.

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