**Universidad de Cantabria**

**Facultad de Medicina**

**Departamento de Biología Molecular**


# Control de la conjugación bacteriana

**María Getino Redondo**

**Santander, 2016**

**University of Cantabria**

**School of Medicine**

**Molecular Biology Department**


# Control of bacterial conjugation

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**Fernando de la Cruz Calahorra**, Catedrático de Genética de la Facultad de Medicina de la Universidad de Cantabria,

**CERTIFICA:** que **María Getino Redondo** ha realizado bajo mi dirección el trabajo que lleva por título "Control de la conjugación bacteriana".

Considero que dicho trabajo se encuentra terminado y reúne los requisitos necesarios para su presentación como Memoria de Doctorado, al objeto de poder optar al grado de Doctor.

Santander, a 1 de Abril de 2016

Fdo. Fernando de la Cruz Calahorra

El presente trabajo ha sido realizado entre el Departamento de Biología Molecular de la Universidad de Cantabria y el Departamento de Microbiología y Genómica del Instituto de Biomedicina y Biotecnología de Cantabria, bajo la dirección del Dr. Fernando de la Cruz Calahorra y gracias a una beca predoctoral de la Universidad de Cantabria.

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*A mi familia*

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Gracias a todos.

María

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## Abbreviations

| | |
|---|---|
| **2,6-HDA** | 2,6-Hexadecadiynoic acid |
| **2,9-HDA** | 2,9-Hexadecadiynoic acid |
| **2-AFA** | 2-Alkynoic fatty acid |
| **2-DDA** | 2-Dodecynoic acid |
| **2-HDA** | 2-Hexadecynoic acid |
| **2-HDOH** | 2-Hexadecyn-1-ol |
| **2-HDOTHP** | 2-(2-Hexadecynyloxy)-tetrahydro-2H-pyran |
| **2-ICA** | 2-Icosynoic acid |
| **2-ODA** | 2-Octadecynoic acid |
| **2-ODOH** | 2-Octadecyn-1-ol |
| **2-ODOTHP** | 2-(2-Octadecynyloxy)-tetrahydro-2H-pyran |
| **2-TDA** | 2-Tetradecynoic acid |
| ***α*** | Growth rate |
| ***δ*** | NMR chemical shift |
| ***γ*** | Conjugation rate |
| ***τ*** | Generation time |
| **AbR/Ab$^r$** | Antibiotic resistance |
| **ACP** | Acyl carrier protein |
| **AHL** | Acylhomoserine lactone |
| **A. L. U.** | Arbitrary light units |
| **AMR** | Antimicrobial resistance |
| **AP** | Accessory proteins |
| **Ap** | Ampicillin |
| **APCI** | Atmospheric pressure chemical ionization |
| **Ard** | Alleviation of restriction of DNA |
| **ATCC** | American type culture collection |
| **ATP** | Adenosine triphosphate |
| **ATPase** | Adenosine triphosphatase |
| ***b*** | Burden |
| **BHR** | Broad host range |
| **bp** | Base pairs |
| **BSA** | Bovine serum albumin |
| **cAMP** | Cyclic adenosine monophosphate |
| **Cas** | CRISPR-associated genes |
| **CECT** | Spanish type culture collection |
| **CF** | Conjugation frequency |

| | |
|---|---|
| **Cm** | Chloramphenicol |
| **COSY** | Correlated spectroscopy |
| **CRISPR** | Clustered regularly interspaced short palindromic repeats |
| **crRNA** | CRISPR RNA |
| **COIN** | Conjugation inhibitor |
| **d** | Donor |
| **DHCA** | Dehydrocrepenynic acid |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **dsDNA** | Double-stranded DNA |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **Eex** | Entry exclusion |
| **ESI** | Electrospray ionization |
| **EtOAc** | Ethyl acetate |
| **Fin** | Fertility inhibition |
| ***fip*** | Fertility inhibition of IncP plasmids |
| ***fiw*** | Fertility inhibition of IncW plasmids |
| **GFP** | Green fluorescence protein |
| **gHMBC** | Heteronuclear multiple-bond correlation spectroscopy |
| **gHSQC** | Heteronuclear single quantum coherence spectroscopy |
| **Gm** | Gentamycin |
| **HGT** | Horizontal gene transfer |
| **HTC** | High-throughput conjugation |
| **IC** | Inhibitory concentration |
| **ICE** | Integrative conjugative element |
| **IHF** | Integration host factor |
| **Inc** | Incompatibility group |
| **IPTG** | Isopropyl β-D-1-thiogalactopyranoside |
| **Km** | Kanamycin |
| **LB** | Luria Bertani |
| **LF** | Lethal factor |
| **Log/Log10** | Decimal logarithm |
| **LPS** | Lipopolysaccharide |
| **MeO 2-HDA** | Methyl 2-hexadecynoate |
| **MeO 2-ODA** | Methyl 2-octadecynoate |
| **MeOH** | Methanol |
| **MF** | Mobilization frequency |
| **MGE** | Mobile genetic element |

| | |
|---|---|
| **MOB** | Mobility |
| **MPF** | Mating pair formation |
| **mRNA** | Messenger RNA |
| **MS** | Mass spectrometry |
| **MTase** | Methyltransferase |
| ***N*** | Total number of cells |
| **NADH** | Nicotinamide adenine dinucleotide |
| **NHR** | Narrow host range |
| **NMR** | Nuclear magnetic resonance |
| **NOESY** | Nuclear Overhauser effect spectroscopy |
| **Nx** | Nalidixic acid |
| **$OD_{600}$** | Optical density (600 nm) |
| ***oriT*** | Origin of transfer |
| ***osa*** | Oncogenic suppression activity |
| **PA** | Protective antigen |
| **PAM** | Protospacer adjacent motif |
| **PC** | Plate-conjugation |
| **PEC** | Profiling of *E. coli* chromosome |
| ***pif*** | Phage interference function |
| ***psi*** | Plasmid SOS interference |
| **pTi** | Tumor-inducing plasmid |
| **r** | Recipient |
| **R** | Relaxase |
| **REase** | Restriction endonuclease |
| **Rif** | Rifampicin |
| **RM** | Restriction-modification |
| **RNA** | Ribonucleic acid |
| **RNase** | Ribonuclease |
| **rRNA** | Ribosomal RNA |
| **scFv** | Single chain variable fragment |
| **SD** | Standard deviation |
| **Sm** | Streptomycin |
| **ssDNA** | Single-stranded DNA |
| **ssRNA** | Single-stranded RNA |
| **T-strand** | Transferred strand |
| **T3SS** | Type III secretion system |
| **T4CP** | Type IV coupling protein |
| **T4SS** | Type IV secretion system |

| | |
|---|---|
| **T6SS** | Type VI secretion system |
| **Tc** | Tetracycline |
| ***tir*** | Transfer inhibition of RP4 |
| **Tp** | Trimethoprim |
| **TZA** | Tanzawaic acid |
| **UDP** | Uridine diphosphate |
| **uFA** | Unsaturated fatty acid |
| **wt** | Wild type |
| ***x*** | Number of plasmid-free cells |
| ***y*** | Number of plasmid-containing cells |

# Introduction

## 1. Antibiotic resistance

Antibiotics have saved the lives of countless people that suffered from bacterial infections since Alexander Fleming discovered penicillin in 1928 (Fleming 1929). Nevertheless, this success was accompanied by the emergence of antibiotic resistance (AbR). It is thought that AbR arose originally as a self-protection mechanism of the producer organisms (D'Costa *et al.* 2006). AbR genes rapidly disseminated through the biosphere, as a result of a large selection pressure from human application of antibiotics (Davies and Davies 2010). In the case of penicillin, even before its introduction as a therapeutic agent, an enzyme capable of destroying it, called penicillinase, was identified in *Escherichia coli* (Abraham and Chain 1940). As penicillinase, new resistance mechanisms capable of rendering new discovered drugs ineffective emerged with astonishing speed, rapidly reaching human pathogens and increasingly invalidating newer antimicrobial therapies. A schematic timeline of AbR acquisition is shown in **Figure 1**.

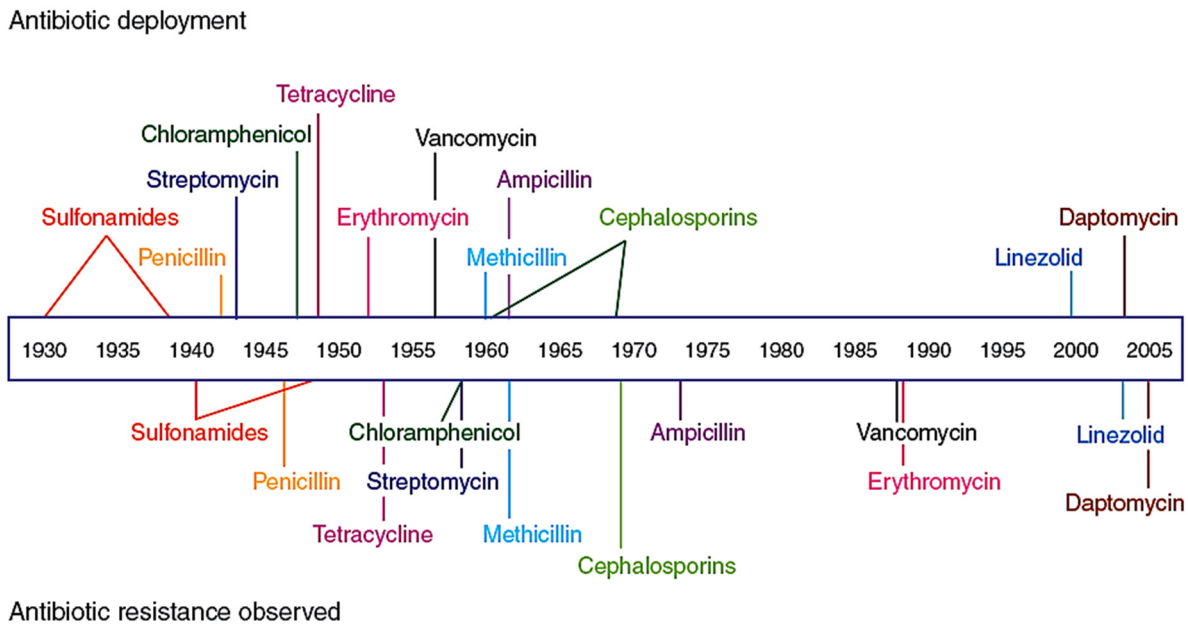



**Figure 1. Timeline of antibiotic deployment and the evolution of AbR.** Few years after a new antibiotic is commercially adopted (above the timeline), clinically significant resistance to that treatment is detected (below the timeline). Taken from (Clatworthy *et al.* 2007).

Penicillinase was the first reported mechanism of AbR (Abraham and Chain 1940). It is a specific β-lactamase, an enzyme able to hydrolyze the penicillin β-lactam ring, preventing its action against bacterial cell-wall synthesis (Sykes and Matthew 1976). In addition to drug inactivation, intracellular targets can also be altered to avoid antibiotic

action. For example, a modified dihydrofolate reductase confers trimethoprim resistance (Amyes *et al.* 1978). A third type of mechanism involves a reduction in the intracellular concentration of the drug. It can occur by the resistance enzyme decreasing membrane permeability, as shown in *Pseudomonas aeruginosa* (Livermore 1992), or by active efflux pumps, to eliminate tetracyclines, chloramphenicol and fluoroquinolones (Williams 1996). Other resistance mechanisms include bypass pathways to avoid inhibited steps or target overproduction. A summary of all these mechanisms is shown in **Figure 2**. Altogether, more than 20,000 potential resistance genes of nearly 400 different types have been predicted from available bacterial genome sequences (Liu and Pop 2009).

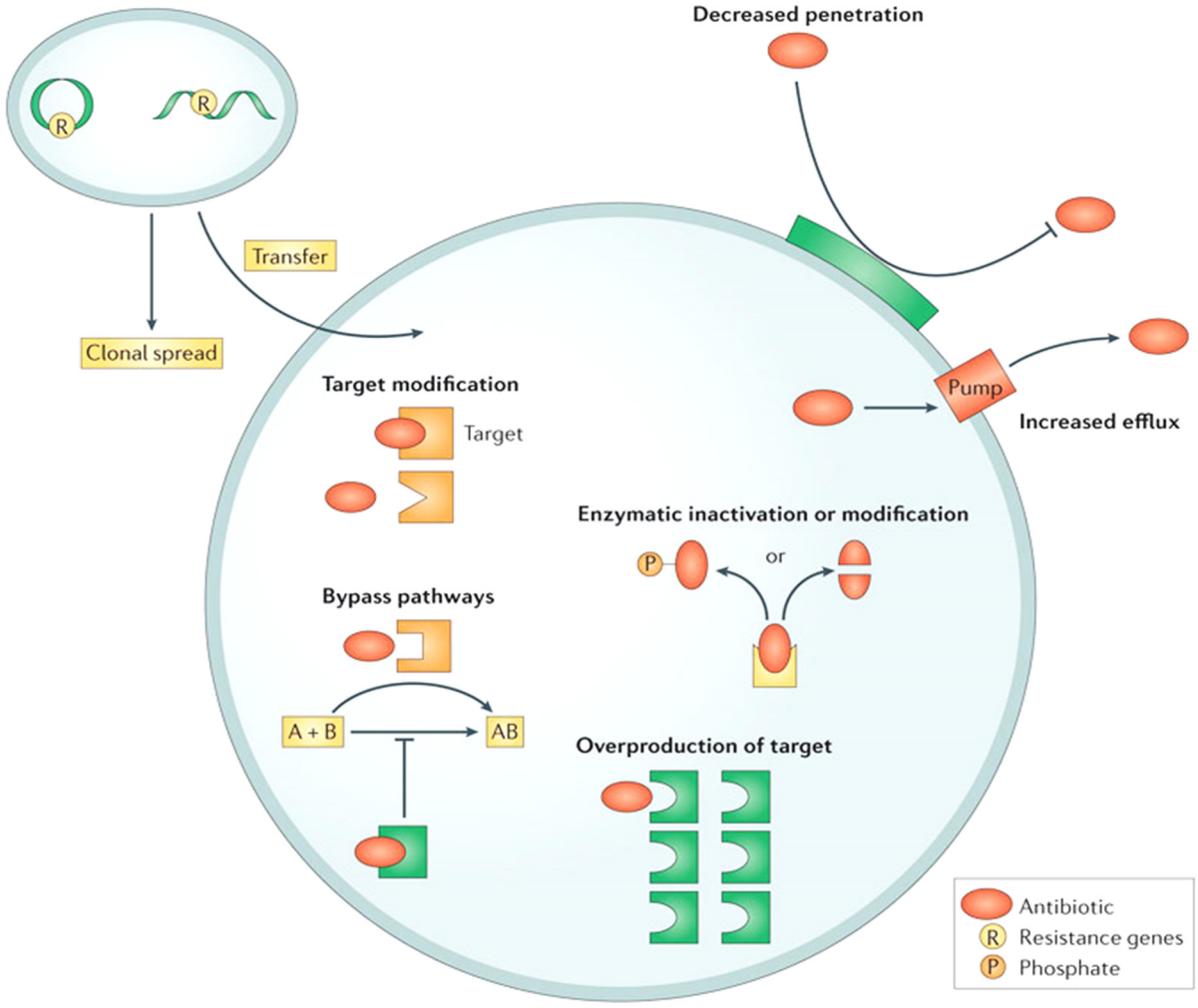



**Figure 2. Mechanisms of genetic resistance to antibiotics.** Bacteria resist antimicrobial action by five main mechanisms, which can be disseminated by horizontal or vertical gene transfer. Target modification: the site where an antibiotic acts, such as the ribosome or the enzyme dihydrofolate reductase, can be altered to avoid its effect. Bypass pathways: bacteria can avoid the blocked step using a different way to produce compound AB. Decreased penetration/increased efflux: intracellular concentration of a drug can be diminished either by reducing membrane permeability to avoid its entry or by actively pumping the antibiotic outside the cell. Enzymatic

inactivation or modification: antibiotics can be altered by specific enzymes, such as β-lactamic antibiotics by β-lactamases, to prevent their action. Overproduction of target: bacteria can produce higher amounts of the target, allowing antibiotic-free targets to act normally. Adapted from (Coates *et al.* 2002).

The danger created by the ever increasing number of pathogens resistant to conventional antibiotics is further increased by the important drop in the development of new antimicrobial compounds, as schematized in **Figure 3**. Development costs are too high for drugs that will be sold mainly for short, acute treatments and to which resistance may rapidly emerge. Instead, drug companies are shifting their research efforts to develop drugs that treat chronic conditions, such as diabetes, obesity, or high blood pressure (Cooper and Shlaes 2011).

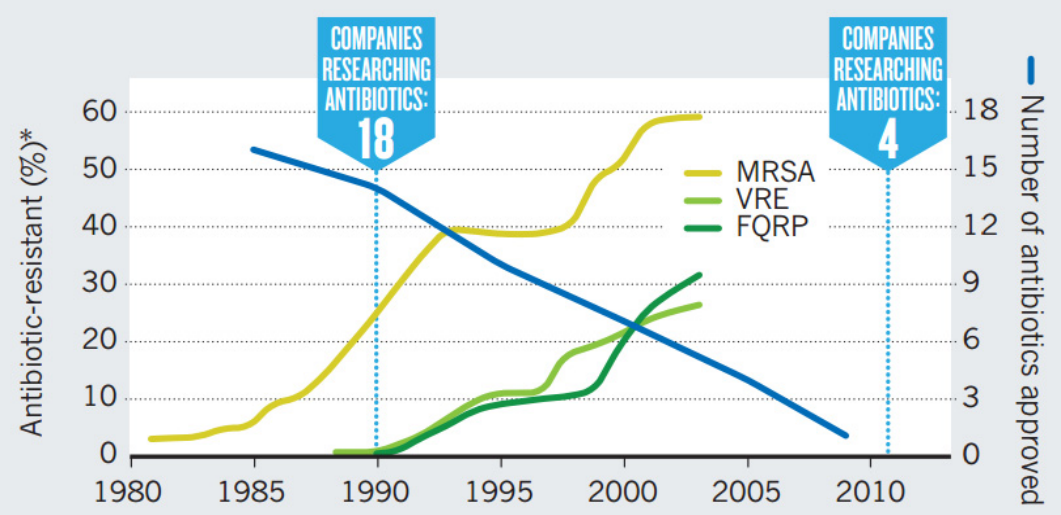



**Figure 3. AbR dual problem.** Growth of bacterial infections resistant to available treatments adds to a decrease in the number of new antibiotics being approved. * Proportion of clinical isolates that are resistant to antibiotics. MRSA, methicillin-resistant *Staphylococcus aureus*. VRE, vancomycin-resistant *Enterococcus*. FQRP, fluoroquinolone-resistant *P. aeruginosa*. Taken from (Cooper and Shlaes 2011).

Nearly 700,000 annual deaths worldwide are currently attributed to antimicrobial resistance, and 50,000 across Europe and the United States alone. Moreover, AbR has been estimated to cost US hospitals more than $20 billion annually (Cooper and Shlaes 2011). This situation demands solutions to avoid that hundreds of thousands people dying each year become millions (**Figure 4**). Some proposed strategies include more accurate prescription policies and a controlled use and release of antibiotics in animal husbandry and agriculture. However, these restrictions are difficult to implement on a global scale (Davies and Davies 2010).

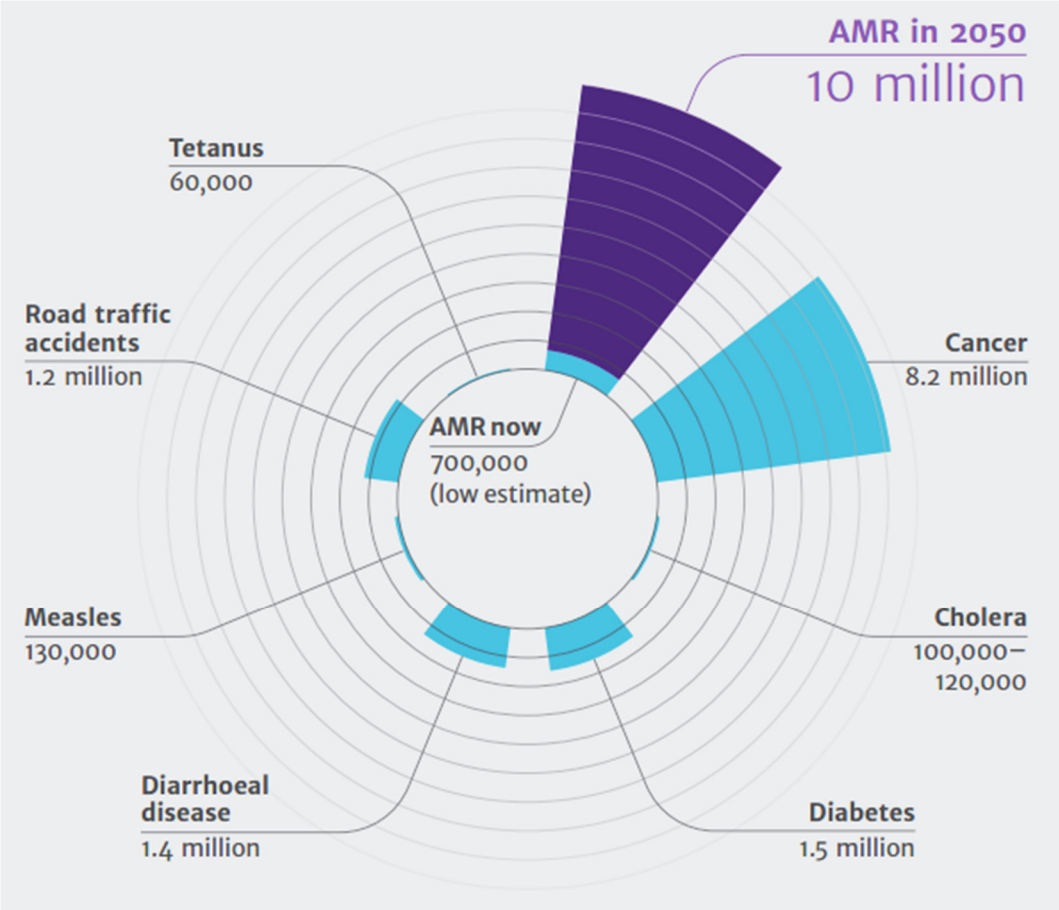



**Figure 4. Estimation of deaths caused by AbR infections.** Worldwide number of deaths attributable to AbR every year compared to other causes of death nowadays (blue), estimated for year 2050 (purple). AMR, antimicrobial resistance. Taken from (O'Neill 2014).

Alternatives to conventional antibiotics are emerging to treat this global crisis. For example, inhibitors of resistance mechanisms, such as efflux pumps (Lomovskaya *et al.* 2007) or β-lactamase inhibitors (Reading and Cole 1977), in combination with antibiotics. However, bacteria evolve rapidly to counteract them and β-lactamases resistant to clavulanic acid have also appeared (Thomson and Amyes 1992). Inhibitors of bacterial virulence are promising alternatives with an advantage over antibiosis in that selection for resistance might not occur because the growth of the pathogen would not be impaired (Heras *et al.* 2015). Thus, anti-toxin compounds (Shoop *et al.* 2005), adhesion inhibitors (Pan *et al.* 2005, Pinkner *et al.* 2006), or transcriptional regulators of virulence genes (Hung *et al.* 2005), including quorum quenchers (Tang and Zhang 2014), are some of the strategies proposed to block virulence (**Figure 5**).

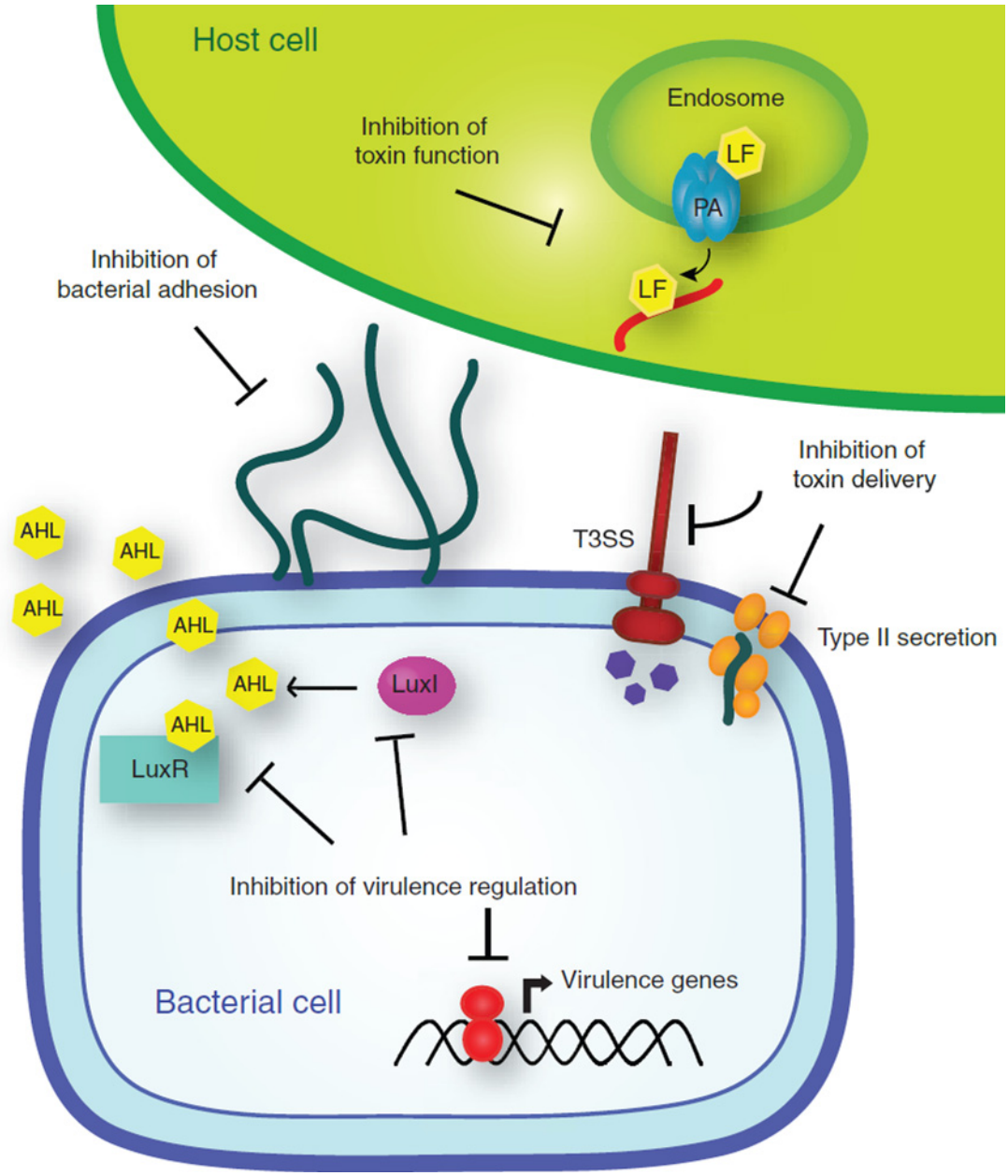



**Figure 5. Inhibitors of bacterial virulence.** Virulence inhibitors can target different bacterial functions to prevent damage of the eukaryotic host cell. Inhibition of toxin function: the activity of toxic proteins secreted by pathogenic bacteria, such as the lethal factor (LF) of *Bacillus anthracis*, can be disrupted in the host cell using inhibitors of its protease activity or its translocation from the endosome through the protective antigen (PA). Inhibition of bacterial adhesion: adhesion step to host cells can be inhibited by pilicides, molecules that block pilus assembly through targeting pilin chaperones. Inhibition of toxin delivery: toxin release can be blocked by targeting bacterial secretion systems, such as type II or type III secretion systems (T3SS) of *Yersinia* spp. Inhibition of virulence regulation: transcription of virulence genes can be repressed to prevent toxin production by interfering with quorum sensing process, specifically with acylhomoserine lactone (AHL) signaling molecules, their synthesis by LuxI, or their sensing by the activator LuxR. Taken from (Clatworthy *et al.* 2007).

Additional developing lines of attack are inhibitors of plasmid replication and stability (Thomas and Nielsen 2005, Williams and Hergenrother 2008, Baquero *et al.* 2011), antimicrobial peptides (Guilhelmelli *et al.* 2013) and bacteriocins (Cavera *et al.* 2015), inhibitors of iron metabolism (Foley and Simeonov 2012), antimicrobial polymers (Jain *et al.* 2014), enzybiotics (Hermoso *et al.* 2007), antisense antibiotics (Woodford and Wareham 2009), antimicrobial nanoparticles (Beyth *et al.* 2015), light based anti-infectives (Yin *et al.* 2013), vaccines (Scully *et al.* 2015), probiotics (Amara and Shibl 2015), or phage therapy (Nobrega *et al.* 2015), among others.

## 2. Horizontal gene transfer

AbR genes are either vertically transferred from one bacterium to another by clonal expansion or laterally through horizontal gene transfer (HGT), a great source of bacterial variability. HGT is mediated by mobile genetic elements (MGEs), that is, DNA devices for the intra- or intercellular movement of DNA. Intracellular mobility is produced by transposons, DNA fragments with the ability of moving through specific mechanisms of recombination from one genome location to another, including different replicons of the same cell (Frost *et al.* 2005). Intercellular mobility occurs by one of the three main processes: transformation, conjugation, or transduction (**Figure 6**).

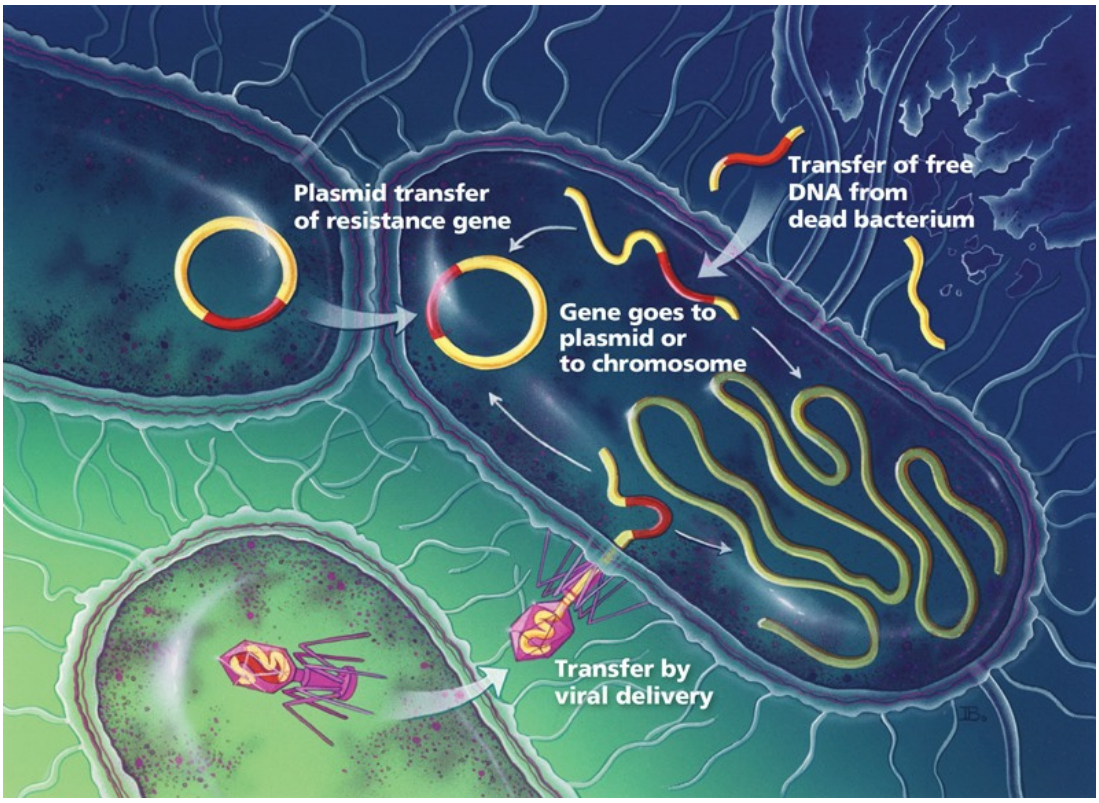



**Figure 6. HGT of AbR genes.** Conjugation, transformation and transduction are the three main mechanisms of horizontal transfer involved in the dissemination of resistance genes. Conjugation consists of plasmid transfer from a donor to a recipient bacteria through direct contact. Transformation involves free DNA uptake by competent bacteria released from dead cells. Transduction requires bacteriophages that accidentally pack DNA from the bacterial host and inject it into a new host. Once an AbR gene is acquired by a bacteria, it can be inserted in the chromosome or replicate autonomously within a plasmid. Taken from (Potera 2013).

Transformation involves extracellular DNA uptake, integration and functional expression. Bacteria must be in a physiological state of competence to acquire

exogenous DNA, which could be natural or artificially induced. Most naturally transformable bacteria develop competence in response to specific environmental conditions, such as altered growth conditions, nutrient access, cell density, or starvation (Thomas and Nielsen 2005). Conjugation requires genetic elements encoding the apparatus needed for their transfer from a donor to a recipient cell through direct contact (Frost *et al.* 2005). Transduction is mediated by bacteriophages, bacterial viruses that accidentally pack segments of host DNA and inject them into a new host, in which they can recombine with the chromosome. Transduction may be generalized or specialized, depending on whether any gene may be transferred or only those located near the site of prophage integration (Davison 1999).

## 2.1. Bacterial conjugation

Conjugation is arguably the most common mechanism of HGT (Halary *et al.* 2010), and the one with the broadest host range (Amabile-Cuevas and Chicurel 1992). Encoded either in autonomously replicating conjugative plasmids or in integrative conjugative elements (ICEs) inserted in the bacterial chromosome, conjugative systems allow the transfer of large DNA fragments containing diverse adaptive traits (Smillie *et al.* 2010). Indeed, they are major vehicles for the spread of AbR genes (Waters 1999, Norman *et al.* 2009).

Bacterial conjugation was discovered by Lederberg and Tatum in 1946, when they mixed two *E. coli* strains with different nutritional requirements and found a third strain with an intermediate phenotype (Lederberg and Tatum 1946, Tatum and Lederberg 1947). To discard cross-feeding between bacteria, Bernard Davis constructed a U-tube with two arms separated by a fine filter too small for bacteria to pass through but large enough to allow passage of metabolites. When he observed that no intermediate phenotype was produced when different strains were grown in each arm, he realized that physical contact was required for this kind of gene transfer (Davis 1950). Conjugation process was described in more detail in 1953, when Watson and Hayes defined the directionality of transfer of the fertility factor F from a $F^+$ donor to a $F^-$ recipient (Watson and Hayes 1953). Two decades later, Datta and Hedges classified plasmids by incompatibility groups (Inc), depending on the ability of two plasmids to coexist stably in the same bacteria (Datta and Hedges 1971). In that same year, F conjugation was nicely photographed by electron microscopy (**Figure 7**).

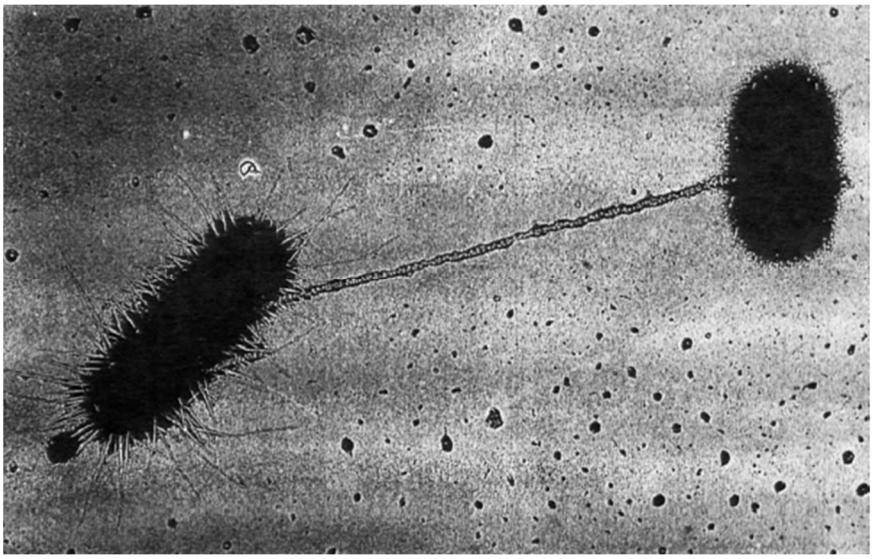


**Figure 7. Electron micrograph of bacterial conjugation.** Donor cell has type I pili (left), while no appendages have observed in recipient bacteria (right). F pilus connecting donor and recipient was covered by M12 RNA phages to make it more visible. Taken from (Brinton 1971).

Either double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) molecules can be transported from donor to recipient cells. Even though dsDNA conjugation was described in Actinobacteria, it requires a single plasmid-encoded septal DNA translocase similar to the segregation ATPase FtsK, unlike the complex machinery needed for "classic" ssDNA conjugation (Thoma and Muth 2015). Conjugative systems involved in ssDNA conjugation carry two sets of genes: mobility (*MOB*) genes for conjugative DNA processing and mating pair formation (*MPF*) genes for DNA delivery through the membranes of donor and recipient bacteria. *MOB* genes include an origin of transfer (*oriT*), a short DNA sequence required in *cis* for plasmid mobility (Furste *et al.* 1989), a relaxase to initiate conjugation and a type IV coupling protein (T4CP) to interconnect DNA processing with DNA transport. *MPF* genes code for a complex of proteins that build the type IV secretion system (T4SS). According to mobility, plasmids can be classified in three categories: conjugative, mobilizable and non mobilizable. A conjugative plasmid contains the two sets of genes necessary for their own transfer, whereas a mobilizable plasmid lacks *MPF* genes and uses the T4SS of a co-resident self-transmissible element, thus escaping from pilus synthesis burden (**Figure 8**). In general, conjugative plasmids tend to be large (> 30 kb) and of low copy number, while mobilizable plasmids are small (< 15 kb) and have high copy number. Plasmids unable to conjugate or mobilize are called non mobilizable (Smillie *et al.* 2010). Nevertheless, non mobilizable plasmids may also be transferred in physical association with a transmissible plasmid by a process called conduction, which requires the co-integration of the transmissible and non

mobilizable plasmids so that transfer of both takes place in a single event (Clark and Adelberg 1962).

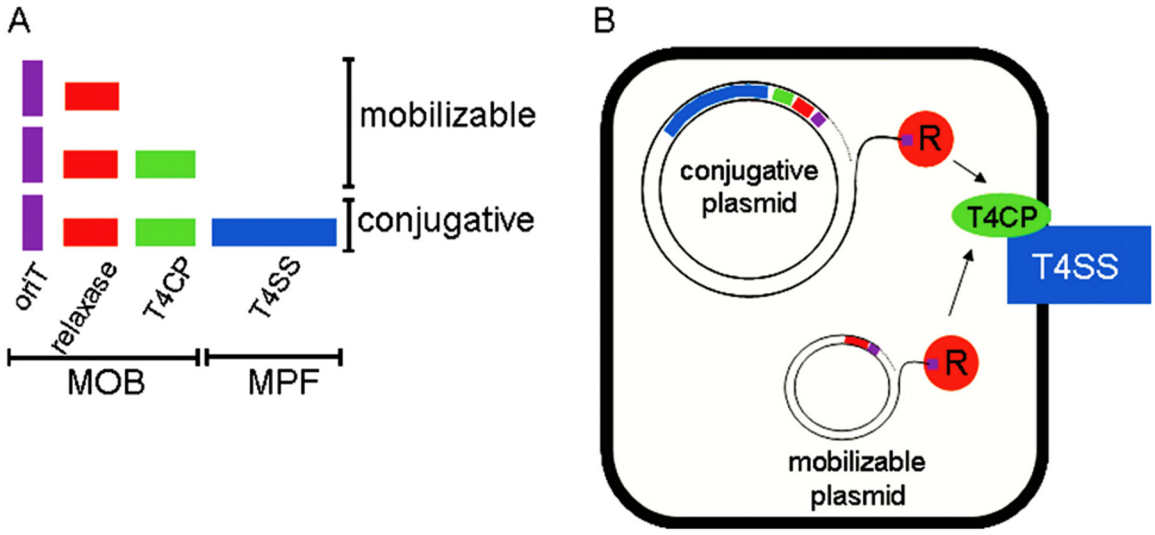



**Figure 8. Essential components of transmissible plasmids.** A) Conjugative plasmids encode *MOB* and *MPF* genes. Mobilizable plasmids contain just a *MOB* module with an *oriT*, a relaxase (R), and an optional T4CP, requiring a compatible MPF (and T4CP) for mobilization. B) The relaxase initiates conjugation by nicking plasmid *oriT*. Covalently bound to plasmid DNA, the relaxase interacts with T4CP and T4SS to be pumped into the recipient cell. Taken from (Smillie *et al.* 2010).

The initial requirement for bacterial conjugation is the expression of *MPF* genes in donor cells. Four MPF classes are found in conjugative systems from Proteobacteria: $MPF_T$ (whose prototype is *Agrobacterium tumefaciens* pTi plasmid), $MPF_F$ (exemplified by conjugative plasmid F), $MPF_I$ (exemplified by IncI plasmid R64) and $MPF_G$ (related to a broad family of ICEs whose prototype is ICE*Hin*1056 of *Haemophilus influenzae*) (Smillie *et al.* 2010). $MPF_T$ class encodes the simplest T4SS, consisting of 11 proteins called VirB1 to VirB11 from *A. tumefaciens* T4SS (Christie *et al.* 2005). The T4SS complex can be divided in four parts: the pilus, the core channel complex, the inner membrane platform and the cytoplasmic ATPases that supply the energy for pilus biogenesis and substrate transport (Low *et al.* 2014). The conjugative pilus is the appendage that extends from the donor cell to reach the recipient cells within its proximity and subsequently retracts it to facilitate cell contact (Clarke *et al.* 2008). Retraction has not been demonstrated for all types of pilus (Cabezon *et al.* 2014). Pilus morphology (rigid, thick flexible, or thin flexible) determines the ability of plasmids to conjugate in liquid media or on solid surfaces (such as biofilms). For example, plasmids that determine rigid pili (Inc groups M, N, P, and W) transfer at least 2,000 times better on plates than in

broth, plasmids with thick flexible pili (Inc groups C, D, F, H, J, T, V, and X) conjugate 45-470 times better on solid media, and plasmids with thin flexible pili (Inc groups I, B, and K) transfer equally well in both situations (Bradley 1980, Bradley *et al.* 1980). This feature, added to plasmid host range (del Solar *et al.* 1996), and pilus implication in establishing bacterial biofilms (Ghigo 2001), are important determinants for plasmid dissemination in the environment (Norman *et al.* 2009).

Once donor-recipient contact is established, the next step in conjugation is DNA processing (**Figure 9**), driven by MOB proteins. Based on MOB sequences and DNA-processing mechanism, transmissible plasmids are classified in six MOB families: $MOB_F$, $MOB_H$, $MOB_Q$, $MOB_C$, $MOB_P$, and $MOB_V$ (Garcillan-Barcia *et al.* 2009).

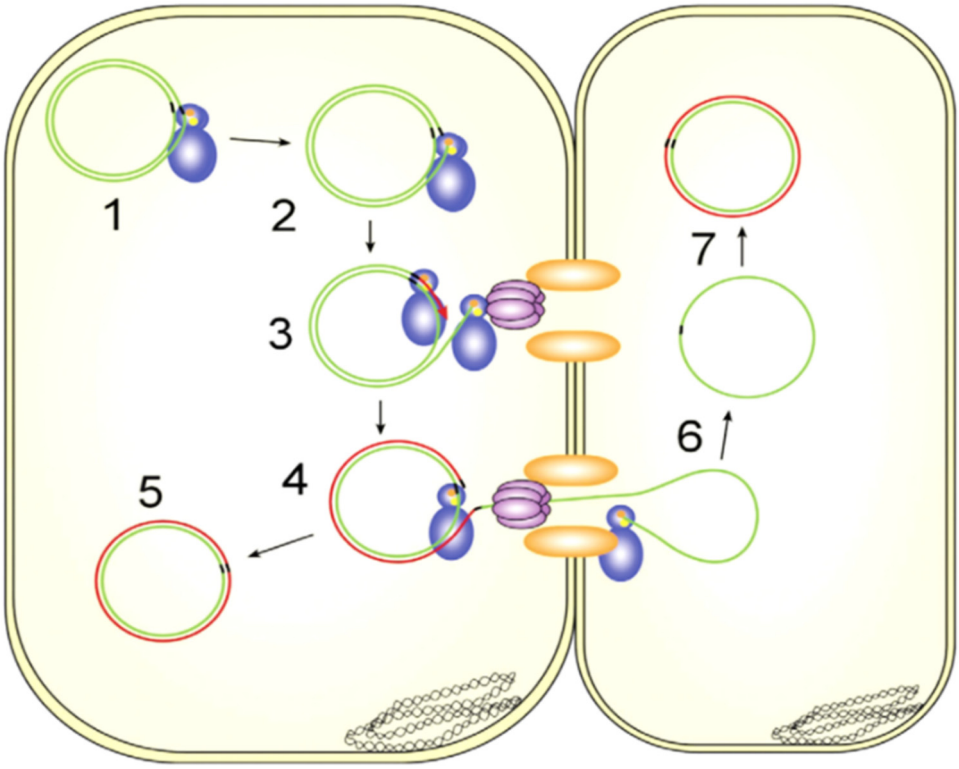



**Figure 9. Model for DNA processing in bacterial conjugation.** 1) TrwC relaxase domain (blue circle) of plasmid R388 binds *oriT*. 2) The relaxase cleaves plasmid DNA at the *nic* site and forms a covalent intermediate with the 5' end of *oriT*. 3) Uncleaved strand is replicated by rolling circle, displacing T-strand. 4) When replication is complete, the relaxase releases T-strand through a second cleavage at the *nic* site. 5) Double-stranded plasmid remaining in the donor is recircularized. 6) The T4CP TrwB (purple) applies the motion force for the complex relaxase-DNA to be transferred into the recipient cell through the T4SS (orange) and the relaxase in the recipient religates T-strand ends. 7) Replication results in a double-stranded plasmid in the recipient cell. Taken from (Williams and Hergenrother 2008).

The key protein for DNA transfer initiation, present in all transmissible plasmids, is the relaxase. It constitutes, with specific auxiliary factors, a nucleoprotein complex on the *oriT* called relaxosome. The relaxase is directed to the *nic* site within the *oriT* by auxiliary factors, DNA binding proteins involved in the regulation of gene expression, such as TrwA and the chromosomally-encoded integration host factor (IHF) of plasmid R388 (de la Cruz *et al.* 2010). At this specific position, the relaxase cleaves the phosphodiester bond of the DNA strand to be transferred (T-strand) via a nucleophilic attack by the hydroxyl group of the catalytic tyrosine residue on the 5' phosphate of DNA (Byrd and Matson 1997). This transesterification reaction results in a covalent linkage between relaxase and ssDNA (Guasch *et al.* 2003), followed by DNA replication from 3' end of the cleaved strand, using the remaining circular strand as a template. A helicase domain, usually present at the C-terminus of the relaxase domain (Garcillan-Barcia *et al.* 2009), unwinds DNA to displace the T-strand (Llosa *et al.* 1996). Once replication is complete, the relaxase produces a second cleavage at the *nic* site to release the T-strand from the newly formed strand (Pansegrau and Lanka 1996).

Once first nicking reaction has ended, T4CP mediates relaxosome recruitment to the T4SS with the assistance of auxiliary proteins (Llosa *et al.* 2002), in order to start the transfer process (**Figure 10**). Although the mechanism of transport across the T4SS channel is still unknown, it is thought that the ATPase VirB11 acts as an acceptor of the relaxosome from T4CP (Cascales and Christie 2004). Then, the nucleoprotein complex is delivered to the inner membrane platform at the base of the T4SS to cross the channel that connects donor with recipient cells (Cabezon *et al.* 2014). According to the shoot and pump model (Llosa *et al.* 2002), once the relaxase is shot through the channel acting as a pilot protein for the T-strand, T4CP pumps remaining ssDNA using the energy derived from ATP hydrolysis. When a complete copy of plasmid ssDNA reaches the recipient cell, the relaxase recognizes the *nic* site as a termination site and carries out the reverse nicking reaction (the free hydroxyl group of DNA attacks the 5' end), resolving the covalent intermediate relaxase-DNA and resulting in re-circularization of T-strand in the recipient cell (Draper *et al.* 2005, Garcillan-Barcia *et al.* 2007). Finally, a second strand is synthesized by rolling-circle replication to generate a copy of the original conjugative plasmid in the recipient cell, thus turning it into a new donor.

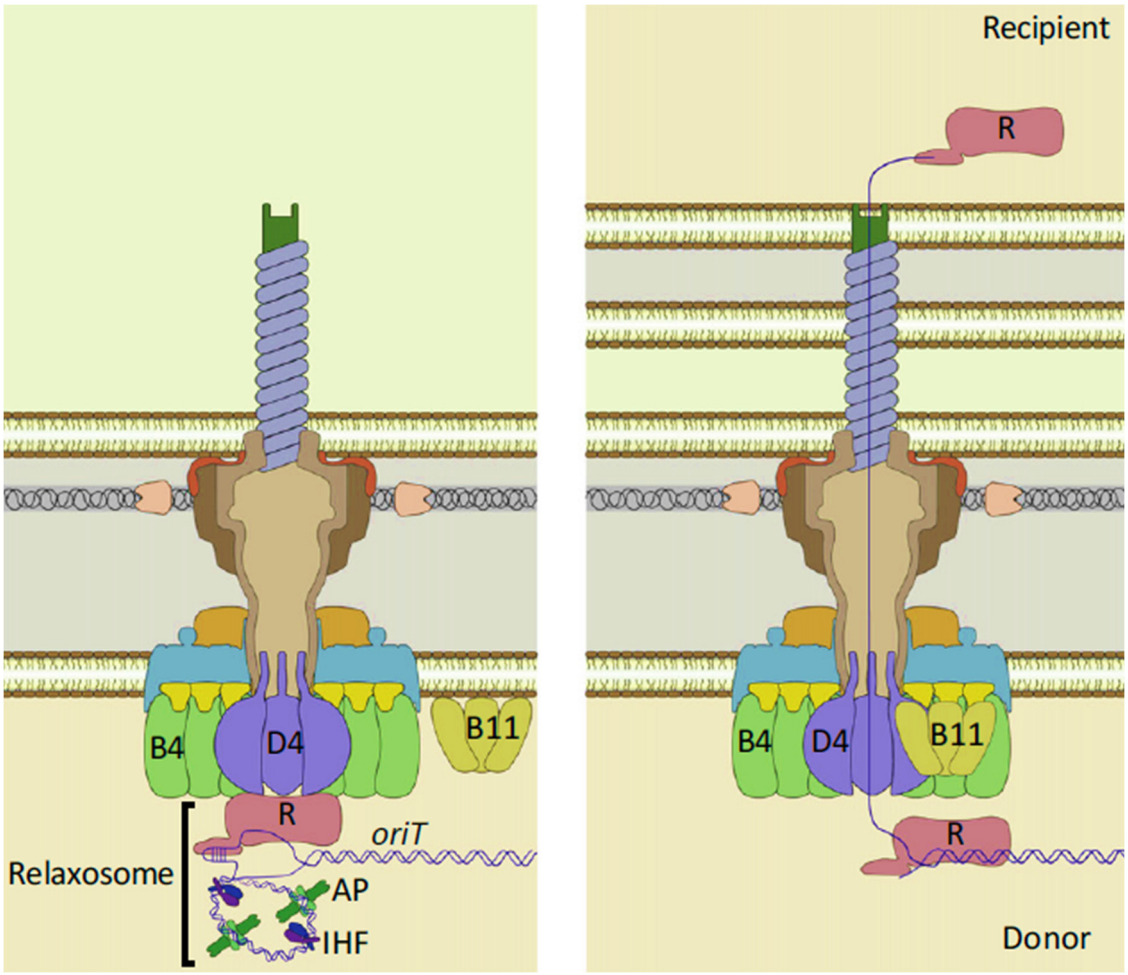



**Figure 10. Model for T4SS substrate transport.** VirB11 protein interacts with T4CP (VirD4) to switch from pilus biogenesis mode to substrate transfer mode. The relaxase (R) and accessory proteins (AP and IHF) bound to the *oriT* integrate the relaxosome, which is recruited to the T4SS through interactions with VirD4 (left panel). Then, VirD4 pumps the T-strand piloted by the relaxase to the recipient cell via T4SS (right panel). Taken from (Ilangovan *et al.* 2015).

# 3. Natural barriers to conjugation

Conjugation is a key mechanism involved in AbR dissemination (Halary *et al.* 2010, Norman *et al.* 2009). For this reason, both natural and artificial barriers to control this process will be discussed. First, the main barriers that bacteria naturally impose to conjugative plasmid propagation will be described.

## 3.1. Host barriers

Natural barriers to conjugation can be found in the host chromosome (host barriers) or in the plasmid genome (plasmid barriers). Among host barriers, restriction-modification (RM) and CRISPRs-Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated genes) systems are the most common mechanisms to inhibit foreign DNA entry in bacteria.

### 3.1.1. Restriction-modification systems

Restriction was first observed in the 1950s when bacteriophage λ, propagated in *E. coli* B, was found to grow poorly on *E. coli* K-12 (Bertani and Weigle 1953). RM systems code for an extremely diverse group of enzymes, ubiquitous among prokaryotes, and involved in defense against invading genomes, such as phages or plasmids (Roberts *et al.* 2015). They comprise two opposing enzymatic activities, restriction endonuclease (REase) and methyltransferase (MTase) (**Figure 11**).

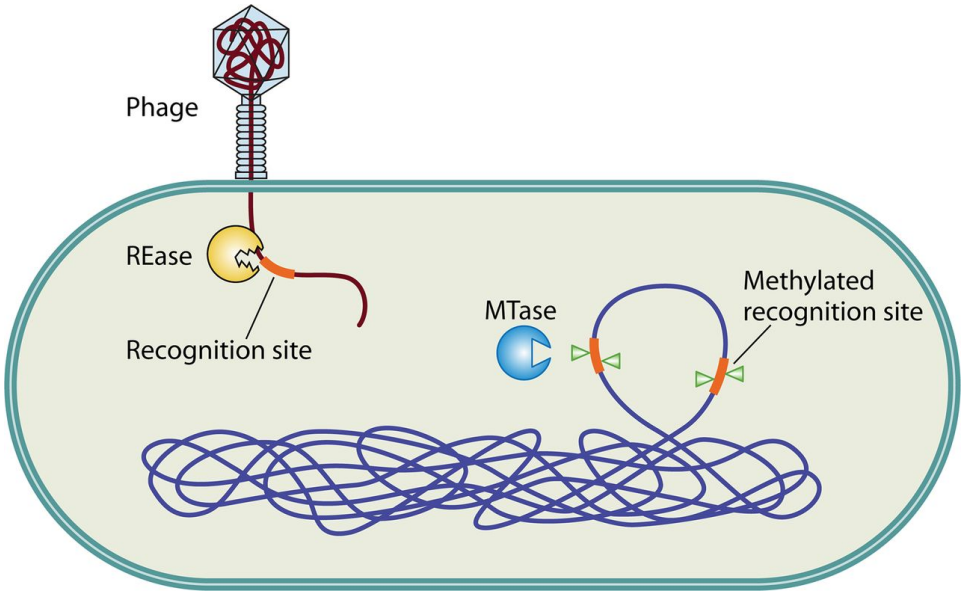


**Figure 11. Schematic mechanism of defensive RM systems.** The methylation status of incoming DNA is recognized by RM systems as foreign. Self-methylation status is maintained by the MTase of the RM system. Recognition sequences on the foreign DNA lacking methylation are cleaved by the REase. Taken from (Vasu and Nagaraja 2013).

The REase recognizes and cleaves foreign DNA at a specific site, whereas the MTase confers protection from cleavage to host genome by methylating a defined adenine or cytosine residue within the specificity site. Due to their ability to recognize self from non-self DNA, RM systems are considered to function as a primitive, innate immune system (Bickle 2004). They are classified mainly into four different types based on their molecular structure, sequence recognition, cleavage position, and cofactor requirements: type I, II, III, and IV (Roberts *et al.* 2003).

RM systems are major players in the co-evolutionary interaction between MGEs and their hosts (Oliveira *et al.* 2014). In addition to host protection against invading phages and other genetic elements, RM systems may have additional roles (Vasu and Nagaraja 2013). For example, MGE-encoded RM systems can act as toxin-antitoxin stability systems. During cell division, the failure to segregate RM systems efficiently results in post-segregational killing of the progeny lacking the RM-containing plasmids. This is due to the higher stability of the REase (toxin), which attacks the unmodified host genome of the progeny lacking the MTase (antitoxin) (Kobayashi 2001). Thus, the MGE is stabilized by the RM system and the RM system acquires the ability of being transferred. Although this role contributes to the stability of RM-containing plasmids instead of being a barrier to conjugation, it seems to be a minor role, since only 10.5 % of the plasmids encode RM systems, whereas 69 % of the chromosomes do so (Oliveira *et al.* 2014).

While host defense against bacteriophage infection has been extensively described (Samson *et al.* 2013), inhibition of plasmid conjugation by RM systems has been reported to a lesser extent. Among these works, some revealed that inactivation of restriction systems in recipient cells (Roer *et al.* 2015, Waldron and Lindsay 2006, Pinedo and Smets 2005, Geisenberger *et al.* 1999, Schafer *et al.* 1994, Trieu-Cuot *et al.* 1991) or methylation systems in donor cells (Zhou *et al.* 2012) increase conjugation frequency, while others showed a reduction in conjugative transfer when the number of restriction sites in the donor plasmid was increased (Ohtani *et al.* 2008, Purdy *et al.* 2002, Elhai *et al.* 1997). Accordingly, the ability of phages and plasmids to escape restriction highlights the importance of RM systems as defense devices against foreign DNA. The mechanisms used in this co-evolutionary arms race between bacteria and parasitic DNA molecules to avoid restriction include different strategies (**Table 1**).

**Table 1**. **Anti-restriction strategies.**

| Mechanism | Anti-restriction strategy | Examples | References |
|---|---|---|---|
| Incoming genome modification | Reduction or re-orientation of restriction sites | T3 or T7 phages | (Kruger *et al.* 1988, Meisel *et al.* 1992) |
| | Incorporation of unusual bases or methylation | Mu or SPβ phages | (Warren 1980) |
| Restriction sites occlusion | Transient occlusion of restriction sites by proteins co-transported with the DNA | P1 phage DarA/DarB | (Iida *et al.* 1987) |
| Host RM systems alteration | MTase stimulation to modify incoming DNA | λ phage Ral protein | (Zabeau *et al.* 1980) |
| | Destruction of REase cofactors | T3 phage SAMase | (Studier and Movva 1976) |
| REase inhibition | Direct inhibition of restriction enzymes through mimicking DNA size, shape, and electric charge | T7 phage Ocr protein | (Walkinshaw *et al.* 2002) |

Phages and plasmids avoid bacterial restriction defenses using four main mechanisms (Tock and Dryden 2005).

A number of conjugative plasmids encode anti-restriction proteins, named Ard (alleviation of restriction of DNA). ArdA and ArdB, encoded by transposons and plasmids of IncN, IncI, and IncF groups, are examples of direct inhibitors of REases that mimick DNA structure after their rapid expression in recipient cells (McMahon *et al.* 2009, Wilkins 2002). ArdC protein from IncW plasmid pSa protects incoming T-strand by transient occlusion of restriction sites after being pumped into recipient cell (Belogurov *et al.* 2000). Another strategy is the selection of plasmid variants that have lost most restriction sites, as seems to have happened in the case of plasmid RP4 (Wilkins *et al.* 1996).

### 3.1.2. CRISPR-Cas systems

Additional defense systems, sometimes operating synergistically with RM systems, are CRISPR-Cas systems (Dupuis *et al.* 2013). Unlike RM systems, which provide a primitive innate immunity to prokaryotic organisms, CRISPR-Cas systems can be thought as involved in adaptive immunity, sequence-directed against foreign elements (Barrangou *et al.* 2007). CRISPR loci, present in 45 % of bacterial and 84 % of archaeal sequenced genomes (Grissa *et al.* 2007), consist of an array of repetitive sequences of 30-40 bp, partially palindromic, and interspersed by equally short spacer sequences of viral or plasmid origin. The acronym CRISPR-Cas stands for clustered, regularly interspaced short palindromic repeats and CRISPR-associated genes (Jansen *et al.*

2002). CRISPR-Cas defense process can be divided into two main phases: immunization and immunity (**Figure 12**).

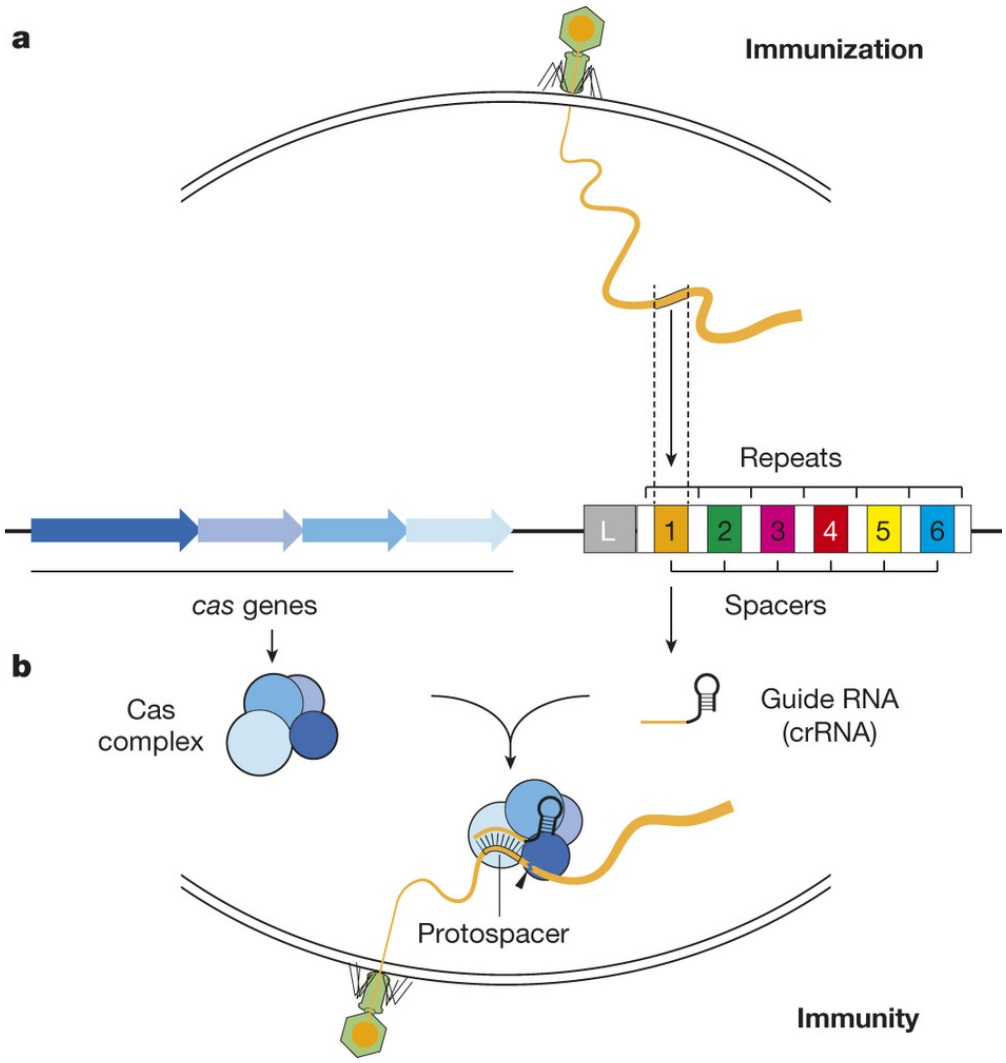



**Figure 12. Overview of the CRISPR-Cas mode of action.** a) Immunization. When DNA from a virus or a plasmid reaches a bacterial cell, the Cas complex cleaves foreign DNA and integrates a repeat-spacer unit at the promoter-containing leader end (grey box) of the CRISPR array. b) Immunity. The CRISPR array is transcribed into pre-crRNA, which is processed into mature crRNA. A crRNA acts as an antisense guide for the Cas complex to bind and then cleave the corresponding protospacer at the invading DNA. Taken from (Marraffini 2015).

In the immunization phase, also known as adaptation or spacer acquisition, sequences from the invading genome are integrated into the CRISPR array. The rapid acquisition of new spacers provides an efficient response against phages that escape immunity by mutating the target site (Andersson and Banfield 2008). In the immunity phase, immunity is accomplished in two steps: guide RNA biogenesis, where a CRISPR array is transcribed and processed to generate small CRISPR RNAs (crRNAs), and targeting, in which the spacer present in the crRNA serves as a guide to direct the

cleavage of the complementary sequence at the invading DNA (protospacer) by the Cas nucleases.

Bacteria must distinguish between protospacers of invading genomes and spacers of their CRISPR arrays to avoid cleavage of their own chromosome (Marraffini and Sontheimer 2008). CRISPR-Cas systems can be classified into three types, based on their Cas content, crRNAs biogenesis mechanism, and targeting requirements (Makarova *et al.* 2011). In type I and II systems, autoimmunity is prevented through a sequence called protospacer adjacent motif (PAM), only present in the invading DNA, upstream the protospacer. The presence of this sequence is essential for foreign DNA cleavage by Cas nucleases (Sashital *et al.* 2012). No PAM requirements have been described in type III systems. Therefore, autoimmunity inhibition is thought to occur through detection of differential base pairing between crRNA and protospacer, preventing cleavage when full complementarity is detected (Marraffini and Sontheimer 2010). In addition, Chi sites (8-nucleotide motifs highly enriched in bacterial genomes) limit the acquisition of chromosomal fragments, favoring the acquisition of foreign elements, also more likely fragmented during replication (Levy *et al.* 2015). A failure in autoimmunity prevention leads to host death, a consequence that has been exploited for the use of CRISPR-Cas systems as genome editing tools both in prokaryotes and eukaryotes (Sontheimer and Barrangou 2015, Jiang *et al.* 2013).

Among the numerous emerging applications of CRISPR-Cas systems (Pennisi 2013), their ability to attack plasmid DNA during conjugation locates them as new weapons against AbR dissemination. In their first work, Marraffini and Sontheimer showed that a spacer from a clinical isolate of *Staphylococcus epidermidis*, which matched a region of the nickase gene present in staphylococcal conjugative plasmids, prevented conjugation and transformation of plasmids containing this sequence (Marraffini and Sontheimer 2008). Moreover, the study revealed that the CRISPR-Cas target was DNA instead of RNA by placing a self-splicing intron in the nickase target sequence. In this line of research, the analysis of CRISPR spacers related to conjugative plasmids revealed that protospacers are not randomly distributed, but display a MOB family-dependent bias. Whereas $MOB_P$ plasmids are usually targeted within the lagging regions, protospacers of the $MOB_F$ family are mostly located in the leading region (the first plasmid section entering the recipient cell). Nevertheless, when conjugation of the $MOB_F$ plasmid F was inhibited using a type I CRISPR-Cas system, the level of protection was independent of the protospacer position and the DNA strand, suggesting that the observed bias depends on the spacer acquisition phase or on the first regions becoming double-stranded (Westra *et al.* 2013). A few additional studies demonstrate the conjugation interfering role of CRISPR-Cas systems (Samson *et al.* 2015). Some described interference in

*Listeria monocytogenes* (Sesto *et al.* 2014), *S. epidermidis* (Hatoum-Aslan *et al.* 2014), or *Pectobacterium atrosepticum* (Richter *et al.* 2014). In addition to plasmid transfer inhibition, spacers of plasmid origin could target AbR genes to induce plasmid loss (Garneau *et al.* 2010) or even trigger AbR pathogen death (Bikard *et al.* 2014, Yosef *et al.* 2015), among other interesting alternatives with countless possibilities.

### 3.1.3. Other host factors involved in conjugation

Recent discoveries of new defense systems against phage infection and plasmid transformation might be involved in protection against plasmid conjugation too. This is the case for prokaryotic Argonaute proteins, homologs to the eukaryotic nucleases involved in RNA interference (Makarova *et al.* 2009, Swarts *et al.* 2014), or bacteriophage exclusion, a mechanism that protects bacteria from phage replication (Barrangou and van der Oost 2015, Goldfarb *et al.* 2015).

Besides the previously described defense barriers against incoming DNA, several works aimed to find more host barriers to conjugation or potential targets to control the process. Early studies demonstrated the contribution of the basic cellular machinery (replication, protein synthesis, or energy supply) in bacterial conjugation (Curtiss *et al.* 1968). In particular, DNA polymerase III was shown to be required in recipient cells for the synthesis of the transferred complementary strand (Wilkins and Hollom 1974), as well as in donors to replace the transferred strand (Kingsman and Willetts 1978). Other example is helicase PcrA of *Bacillus subtilis*, needed for ICE*Bs1* DNA unwinding after nicking (Lee *et al.* 2010). Although its homolog UvrD is not essential for *E. coli* growth, PcrA is a second helicase essential for *B. subtilis* viability (Petit *et al.* 1998). Nevertheless, targeting essential enzymes as a barrier to conjugation would kill the host, acting therefore like a conventional antibiotic (Tarantino *et al.* 1999). To avoid the selective pressure that increases the probability of AbR emergence, non-essential functions are preferred to control bacterial conjugation. This is the case of the stationary phase sigma factor RpoS, which regulates ICEclc excision in *Pseudomonas knackmussii* required for its conjugative transfer (Miyazaki *et al.* 2012).

A mechanism potentially deleterious for conjugation as well as for the recipient cell is the SOS response. Sos response is stimulated by the appearance of ssDNA and the RecA protein, which inactivates the LexA repressor thereby inducing several genes involved in DNA repair, recombination, and mutagenesis (Roca and Cox 1997). Some conjugative plasmids are adapted to counteract this response through a plasmid SOS interference (*psi*) system that inhibits RecA binding to ssDNA (Petrova *et al.* 2009). On the other hand, SOS response to DNA damage can also inactivate the LexA repressor homolog

present in several ICEs to induce integrase expression and ICE propagation (Baharoglu *et al.* 2010). Therefore, SOS response can be a positive or a negative regulator of bacterial conjugation.

Host factors involved in the regulation of bacterial conjugation are exemplified by F, a narrow host range (NHR) plasmid, well adapted to *E. coli* (Frost and Koraimann 2010). While broad host range (BHR) plasmids regulate their transfer mostly through plasmid-encoded repressors (Fernandez-Lopez *et al.* 2014), NHR plasmids rely on many host-encoded regulatory factors that act at DNA, RNA, or protein level (**Table 2** and **Figure 13**).

**Table 2**. **Host-encoded factors involved in conjugation of IncF plasmids.**

| Level | Host factor | Regulatory function | References |
|---|---|---|---|
| **DNA** | ArcA/ArcB | Two-component regulatory system that activates transfer in response to oxygen levels | (Serna *et al.* 2010) |
| | SdhABCD | Succinate dehydrogenase has a repressive effect under aerobic conditions, probably by regulating transcription of the activator TraJ | (Serna *et al.* 2010) |
| | Dam | Methylase that modifies certain promoter regions, changing their sensitivity to binding of activators, such as the leucine regulator Lrp to *traJ* promoter | (Camacho *et al.* 2005) |
| | H-NS | Global repressor that silences newly acquire DNA, including transfer genes | (Will *et al.* 2004) |
| | RpoS/RpoH | Alternate sigma factors that stimulate transcription from H-NS silenced promoters | (Shin *et al.* 2005, Wada *et al.* 1987) |
| | FIS | Activator or repressor depending on whether it acts alone or in competition with H-NS | (Dorman 2009) |
| | IHF | Transcriptional activator of transfer genes, besides its primary role as part of the relaxosome architecture | (Gamas *et al.* 1987, de la Cruz *et al.* 2010) |
| | CRP | The cAMP receptor is also a positive regulator of *traJ* expression in response to glucose levels | (Starcic *et al.* 2003) |
| | Unknown | Host-encoded regulator involved in F transfer repression during stationary phase | (Frost and Manchak 1998) |
| **RNA** | RNase E | Ribonuclease that cleaves the antisense RNA FinP (downregulates the translation of the activator TraJ) | (Jerome *et al.* 1999) |
| | Hfq | Global regulator that binds *traJ* mRNA promoting its degradation | (Will and Frost 2006) |
| **Protein** | HslV/HslU | Heat shock protease-chaperone pair involved in TraJ degradation mediated by the two-component system CpxAR in response to extracytoplasmic stress | (Lau-Wong *et al.* 2008) |
| | GroEL | Chaperonin that interacts with TraJ promoting its proteolysis | (Zahrl *et al.* 2007) |

Conjugation of NRH plasmids is regulated by many host factors acting at DNA, RNA, or protein level (Frost and Koraimann 2010).

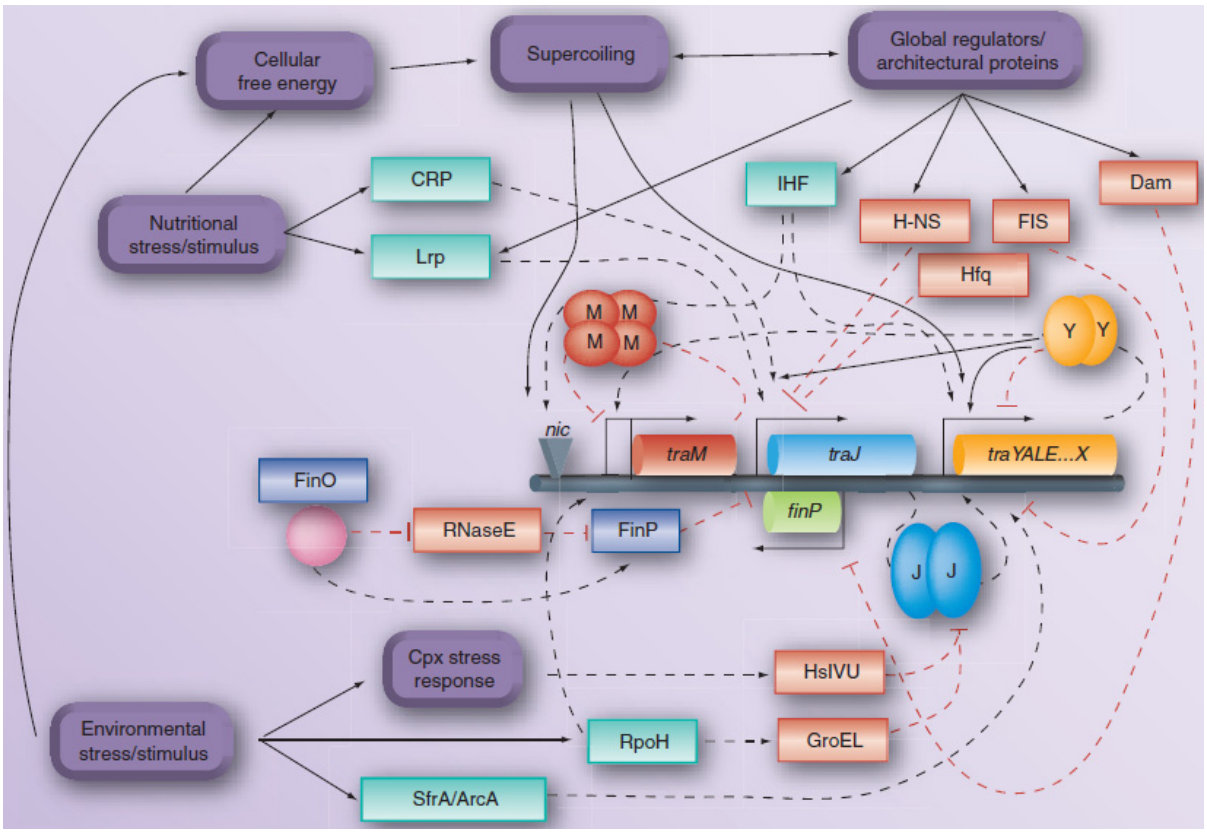



**Figure 13. Regulatory network of IncF plasmids transfer.** Transcripts are shown as arrows arising from their corresponding promoters. F transfer regulatory proteins are represented as tetramers (TraM), dimers (TraJ and TraY), or monomers (FinO). Purple rectangles represent physiological and environmental factors that influence transfer gene expression. Blue rectangles refer to plasmid-encoded regulators, whereas host-encoded regulators with positive and negative effects on transfer potential are denoted by green and red rectangles, respectively. Red dashed lines indicate repression, black dashed lines indicate activation and solid lines indicate generalized effects. Taken from (Frost and Koraimann 2010).

The first systematic screening for host genes involved in conjugation was carried out by Pérez-Mendoza and de la Cruz using two collections of mutants as recipient cells: the Keio collection of 3,908 single-gene deletions and a collection of 20,000 random transposon insertion mutants, which covered more than 99 % of *E. coli* non-essential genome (Perez-Mendoza and de la Cruz 2009). They studied the transfer of the BHR IncW plasmid R388 on solid media through an automated conjugation assay based on the emission of luminescence in transconjugant bacteria. The work indicated that no non-essential recipient genes play a crucial role in conjugation. Therefore, required genes can be either essential for cell growth or redundant. The latter could be the case

for *uvrD* mutant, which showed 41 % of wild type (wt) conjugative transfer. UvrD is a DNA helicase II involved in rolling-circle replication of many plasmids (Bruand and Ehrlich 2000). Despite being an interesting candidate, the slight effect of the mutant in R388 transfer suggested the presence of an alternate helicase. Apart from *uvrD* mutant, only mutations in lipopolysaccharide (LPS) biosynthesis showed a modest decrease in R388 transfer (6 – 32 % of wt), but a more drastic effect was observed on F plasmid liquid transfer, suggesting a role in mating pair stabilization. Accordingly, some previous reports have described different mutants affecting membrane integrity that were defective in recipient ability and have increased susceptibility to antibiotics, detergents, or phages, in particular mutants of LPS biosynthesis and the outer membrane protein OmpA (Watanabe *et al.* 1970, Monner *et al.* 1971, Skurray *et al.* 1974, Havekes *et al.* 1977, Hoekstra and Havekes 1979, Sanderson *et al.* 1981, Manoil and Rosenbusch 1982, Duke and Guiney 1983). Further reports characterized different *rfa* (LPS synthesis) and OmpA mutants during conjugation, proposing the presence of an adhesin at the F pilus tip as the receptor of its specific LPS group (Anthony *et al.* 1994). More recently, recipient LPS was established as the specific receptor for the PilV adhesin of IncI plasmid R64 during liquid conjugation (Ishiwa and Komano 2000) and OmpA was reported to interact with F plasmid TraN for mating pair stabilization (Klimke *et al.* 2005).

Additional approaches using transposon mutagenesis revealed the nitrogen-related phosphotransferase system as the responsible mechanism for conjugation inhibition of IncP-9 naphthalene catabolic plasmid pNAH7 from *E. coli* to *Pseudomonas putida* (Inoue *et al.* 2013). Besides, combining transposon mutagenesis and massive sequencing (van Opijnen and Camilli 2013), Johnson and Grossman searched for recipient functions that contribute to the acquisition of *B. subtilis* ICE*Bs1* by comparing the frequency of each mutation after conjugation in recipient and transconjugant cells (Johnson and Grossman 2014). None of the functions found were essential for ICE conjugation. However, those functions slightly affecting the process were associated with membrane composition, agreeing with previous reports.

Although this thesis is focused on conjugative plasmids from Gram negative bacteria, Gram positive hosts also provide useful data on conjugation control. Gram negative bacteria display a complex T4SS spanning two membranes with a cell-surface attached filamentous pilus. In contrast, Gram positive systems present a minimized T4SS for ssDNA translocation across the single cytoplasmic membrane, with a peptidoglycan hydrolase for local digestion of the cell wall, and adhesins that mediate cell contact (Bhatty *et al.* 2013). The signal for initiating conjugal transfer remains unknown in Gram negative bacteria (Lang *et al.* 2011). On the contrary, many plasmids from Gram positive bacteria use secreted signaling peptides called pheromones to initiate

conjugation (for instance, *Enterococcus faecalis* plasmids pAD1 and pCF10). These pheromones and the machinery needed for their processing and secretion are encoded by the chromosome of recipient bacteria (Dunny 2013). The previously mentioned ICE*Bs1* from *B. subtilis* uses an opposite mechanism that requires the uptake of inhibitory peptides by recipient cells, using a host-encoded oligopeptide permease (Auchtung *et al.* 2005). All these host functions might potentially be targeted to control bacterial conjugation.

## 3.2. Plasmid barriers

Conjugative plasmids display mechanisms that regulate their own transfer, block the entry of related plasmids into the same cell, or inhibit conjugation of plasmids present in the same donor bacteria. Regulatory networks for bacterial conjugation comprise a set of complex responses to maximize successful DNA transport and minimize the burden to cells carrying the conjugative machinery (Koraimann and Wagner 2014). However, plasmid regulatory factors are very diverse between different groups of conjugative systems. To give an example, the regulatory proteins encoded by prototype plasmids F, RP4, and R388 bear no homology relationship whatsoever. Another example of this diversity could be the specific antagonistic signaling of the pair pheromone-inhibitory peptide cCF10-iCF10 in the regulation of the conjugative plasmid pCF10 of *E. faecalis*, a mechanism that has been proposed to control AbR dissemination (Chatterjee *et al.* 2013). Therefore, our analysis of plasmid barriers will focus on entry exclusion and fertility inhibition systems, more conserved between different conjugative plasmids.

### 3.2.1. Exclusion systems

Exclusion phenomenon was first observed when exponentially growing cells harboring F factor were not suitable as conjugation recipients (Lederberg *et al.* 1952). This phenotype, called later "superinfection immunity" (Watanabe 1963), was found to be the combination of two independent mechanisms, plasmid incompatibility and exclusion. Both phenomena referred to an interference between closely related sex factors, the first being associated to replication and the second to conjugation (Novick 1969). F factor was discovered to contain two exclusion systems, surface exclusion and entry exclusion, considered as prototypes for all others. Surface exclusion acts through the outer membrane protein TraT by reducing the ability of recipient cells to form stable mating aggregates (about 10-fold), whereas entry exclusion is based on the recipient inner membrane protein TraS, which inhibits DNA transfer (about 100-fold) after mating pairs have stabilized (Achtman *et al.* 1977, Achtman *et al.* 1979). The precise mechanism of

action remains unclear in both cases. Some hypothesis were put forward to find the TraT receptor in donor cells, which included pilins, a hypothetical adhesin at the pilus tip, or the mating pair stabilization protein TraN. However, none of them was confirmed (Anthony *et al.* 1994, Klimke and Frost 1998). The mechanism of TraS exclusion involves the inner membrane protein TraG in donor cells (Anthony *et al.* 1999). TraG-TraS recognition was later confirmed, proposing that TraG may be translocated into recipient cells for transfer initiation, a process blocked by TraS (Audette *et al.* 2007). However, the donor interacting partner in conjugative plasmids not related to F is unknown, although some TraG-VirB6 similarities point to VirB6 as the TraS counterpart in these systems (Garcillan-Barcia and de la Cruz 2008).

All conjugative plasmids contain at least one exclusion gene, usually TraS-like, indicating their importance for the conjugative element. Exclusion systems may be used to prevent competition among identical plasmid backbones, for donor cells to avoid uneconomical excess of DNA transfer, or for recipient cells to prevent death by lethal zygosis (an excess of cell contacts mediated by pili during conjugation causing extensive membrane damage). Interestingly, only IncF and IncH plasmids, which produce pili that are firmly attached to donor cell, can produce both exclusion systems, while other plasmids whose pili detach easily from the cell, express only entry exclusion (Garcillan-Barcia and de la Cruz 2008).

### 3.2.2. Fertility inhibition

Fertility inhibition was discovered when certain R factors carrying multiple AbR determinants were introduced in cells containing F factor (Watanabe and Fukasawa 1962). These R factors were later revealed as IncFII plasmids producing the protein FinO, which reduced F transfer ability by increasing intracellular levels of the antisense RNA FinP in donor cells (Koraimann *et al.* 1996). FinP RNA specifically downregulates *traJ* mRNA translation, whose product is an activator of the transfer region. FinO binds FinP and *traJ* mRNA helping duplex formation, which triggers *traJ* mRNA cleavage by RNase III and protects FinP from degradation by RNase E (**Figure 13**) (Jerome *et al.* 1999, Jerome and Frost 1999). The F plasmid is naturally derepressed due to *finO* insertional inactivation by the transposable element IS3, resulting in low levels of FinP (Frost *et al.* 1994). Therefore, FinOP fertility inhibition system results in few cells of a bacterial population being transfer-competent, contributing to regulate the balance between conjugative transfer and plasmid burden (including metabolic overhead of constitutive expression and vulnerability to pilus-specific phages) in IncF plasmids. The absence of FinOP regulation in early transconjugant cells produces a transient epidemic

spread that ensures the introduction of a given plasmid in a recipient cell population (Frost and Koraimann 2010).

Besides this auto-regulatory mechanism, which can affect other IncF plasmids due to FinO activity in *trans*, additional fertility inhibition systems have been identified that reduce the conjugative transfer of unrelated co-resident plasmids (Gasson and Willetts 1977). The role of these mechanisms has been suggested to be a better competing ability for colonization of new hosts (Gasson and Willetts 1975). Ten genes from different plasmid groups have been associated with fertility inhibition of plasmids IncF, IncW, IncP, and *A. tumefaciens* pTi (tumor-inducing plasmid) (**Table 3**).

**Table 3**. **Fertility inhibition factors**

| Inhibited | Inhibitors | Mode of inhibition | References |
|---|---|---|---|
| **IncF** | *finQ* / IncI1 | Rho-independent transcription termination at several sites of the *tra* operon | (Gaffney *et al.* 1983) |
| | *finW* / IncFI | Transcription reduction of the activator *traM* | (Gaffney *et al.* 1983) |
| | *finC* / ColE-like | Helper T4CP inhibition during mobilization | (Willetts 1980) |
| | *finU* / IncI1 | Inhibition of *tra* operon transcription and translation/function of a protein involved in pilus assembly | (Gaffney *et al.* 1983) |
| | *finV* / IncX | Inhibition of translation/function of a protein involved in pilus assembly | (Gaffney *et al.* 1983) |
| **IncW** | *fiwA* / IncP1α | Inhibition of translation/function of a protein involved in conjugation | (Fong and Stanisich 1989) |
| | *fiwB* / IncP1α | Inhibition of translation/function of a protein involved in pilus assembly | (Fong and Stanisich 1989) |
| **IncP** | *pifC* / IncFI | Inhibition of T4CP | (Santini and Stanisich 1998) |
| | *fipA* / IncN | Inhibition of T4CP | (Santini and Stanisich 1998) |
| **pTi** | *osa* / IncW | Degradation of T-DNA | (Maindola *et al.* 2014) |

Ten genes encoded by different conjugative or mobilizable plasmids have been identified as conjugation inhibitors of four plasmid groups when present in the same donor bacteria.

As FinOP system, FinQ and FinW systems are thought to act at RNA level, but independently of the main regulator TraJ. FinQ is encoded by IncI1 plasmids (such as R62, R820a, TP102, and TP108) and acts via rho-independent transcription termination at several sites of the *tra* operon (Gasson and Willetts 1975, Gasson and Willetts 1976, Gasson and Willetts 1977, Gaffney *et al.* 1983, Ham and Skurray 1989). FinW is present in IncFI plasmids such as R455 and reduces the transcription of TraM (Gasson and

Willetts 1975, 1977, Gaffney *et al.* 1983), a regulator activated by TraJ and essential for DNA processing during F transfer (Penfold *et al.* 1996). FinC, FinU, and FinV fertility systems act post-transcriptionally. FinC is expressed by the mobilizable plasmid CloDF13 (which uses its own T4CP), probably to inhibit the function of the helper F T4CP during CloDF13 transfer (Willetts 1980). FinU and FinV transfer inhibition genes are encoded by IncI1 plasmid JR66a and IncX plasmid R485, respectively. Since FinU inhibited both pilus assembly and entry exclusion, it was suggested to affect transcription of the *tra* operon (Gasson and Willetts 1975). However, the extent of the transcription reduction was not proportional to the observed effect. For that reason, it was suggested that the primary target of FinU was the translation or function of one or more transfer proteins (Gaffney *et al.* 1983). FinV reduced pilus formation, but did not produce any effect on surface exclusion (Gasson and Willetts 1975). Therefore, it was suggested to act post-transcriptionally, affecting the amount or activity of one of the proteins required for pilus assembly (Gaffney *et al.* 1983).

Genes *fiwA* and *fiwB* encoded by IncP1α plasmids such as RP1 inhibit transfer of IncW plasmids R388, pSa, or pRA3 (Olsen and Shipley 1975, Yusoff and Stanisich 1984, Fong and Stanisich 1989). Both inhibitory functions jointly showed a reduction in R388 conjugation by a factor of $10^{-6}$. While one of them affects only R388 transferability, the other affects also pilus production. Other unidentified mechanisms present in IncX plasmid R6K also inhibited the fertility of IncW plasmid R388 (Olsen and Shipley 1975) and IncN plasmid R46 (Pinney and Smith 1974). Similarly, IncP plasmid RP4 strongly reduced conjugal transfer of the rhizobial plasmids pRmeGR4a and pRmeGR4b (Herrera-Cervera *et al.* 1996).

IncP plasmids are targets of fertility inhibition as well. IncI plasmid R64 encodes a function that inhibits IncP plasmid RP4 conjugation by 10- to 100-fold (Datta *et al.* 1971). IncX plasmid R6K and IncP plasmid RP1 showed reciprocal fertility inhibition through an unknown mechanism that resembled FinOP regulation system (Olsen and Shipley 1975). The first IncN plasmids reported to inhibit IncP plasmids fertility were pN3 (Olsen and Shipley 1975) and R390, which also inhibited IncW plasmid pSa (Coetzee *et al.* 1972). Fertility inhibition of IncP plasmids (or *fip*) was then localized in IncN plasmid pKM101, which reduced RP1 transfer by 10,000-fold (Winans and Walker 1985). The absence of effect in pilus synthesis or entry exclusion suggested that *fip* acted in a different way than FinOP system. An apparently independent function was found in F plasmid that inhibits by 1,000-fold plasmid RP4 conjugation (Tanimoto and Iino 1983). It was located to *pifC* (or *repC*), a gene involved in the initiation of F replication (Tanimoto and Iino 1984) and the regulation of *pif* operon expression (phage interference function) (Miller and Malamy 1983). The protein PifC was responsible of the

inhibition of RP4 conjugation or RP4-assisted mobilization (Miller *et al.* 1985). As occurred with Fip of pKM101, PifC inhibition did not affect exclusion or pilus synthesis. A *pifC* functional homolog named *tir* (transfer inhibition of RP4) was discovered in the replication region of IncF plasmid R100 (Tanimoto *et al.* 1985). The target of FipA and PifC in IncP plasmids was later revealed to be TraG, the T4CP that connects the relaxosome with the T4SS. Both proteins inhibited RSF1010 mobilization (which uses TraG of RP1), while CloDF13 mobilization (which presents its own T4CP) was not affected. In addition, IncN-assisted RSF1010 mobilization enhanced by overexpression of *traG* was lost in the presence of *fipA* or *pifC* (Santini and Stanisich 1998).

IncW plasmid pSa abolished the plant tumor-inducing activity of *A. tumefaciens* when co-resident with pTi plasmid (Loper and Kado 1979, Farrand *et al.* 1981). This suppressive activity was attributed to *osa* gene (oncogenic suppression activity) (Close and Kado 1991). In contrast, the oncogenic suppression caused by IncQ mobilizable plasmids seemed to act by recruiting MPF of pTi plasmid for mobilization and competing for transfer more efficiently than pTi (Ward *et al.* 1991, Binns *et al.* 1995, Stahl *et al.* 1998, Lee and Gelvin 2004). Osa protein shows homology to FiwA from RP1 (Chen and Kado 1994), responsible for IncW fertility inhibition (Fong and Stanisich 1989), and was located at the bacterial inner membrane (Chen and Kado 1996). Osa mode of action was then related to VirE2 export inhibition (Lee *et al.* 1999), a protein involved in T-DNA endonuclease protection and transport (Chumakov 2013). Export of VirE3 and VirF virulence proteins was blocked in the presence of Osa (Schrammeijer *et al.* 2003). Afterwards, IncQ plasmid RSF1010 mobilization by pTi plasmid was confirmed to be reduced by Osa (Lee and Gelvin 2004). By the use of a transfer DNA immunoprecipitation assay, Cascales *et al*. discovered that both IncQ mobilizable plasmids (which use the same pathway than T-DNA) and Osa fertility inhibitor suppressed plant tumorigenesis through the inhibition of T-DNA and VirE2 binding to the T4CP (VirD4) receptor, blocking their passage through *A. tumefaciens* T4SS (Cascales *et al.* 2005). In contrast to IncQ plasmids, which have been proposed to block pTi T4CP as a competing substrate with higher copy number and affinity for T4SS than pTi plasmid (Binns *et al.* 1995), Osa seemed to exert its effects by modulating VirD4 receptor activity through a direct protein-protein interaction. As occurred with FipA and PifC, Osa only inhibited mobilization of plasmids lacking their own T4CP (such as RSF1010), whereas mobilization of plasmids carrying their own T4CP (such as CloDF13) was not affected, thus confirming previous results (Cascales *et al.* 2005). Recently, Osa structure was solved and included in the ParB/Srx superfamily (Maindola *et al.* 2014). ATPase and DNase activities were discovered within its active site, activities that were common to their homologs (fertility inhibition protein ICE1056Fin of *H. influenzae* ICE*Hin*1056 and partition system elements KorB from IncP1α plasmid RK2 and ParB from bacteriophage

P1). In addition, it was shown that T-DNA transfer was also inhibited by Osa homologs ICE1056Fin and FiwA, and even by the unrelated fertility inhibition factors FipA and PifC. Immunoprecipitation and western blot analysis showed Osa interaction with other two T4SS components, VirB4 and VirB11 ATPases. By *in vitro* reconstitution of a partial T4SS (comprising VirB4, VirB11, and Osa), degradation of T-DNA covalently bound to VirD2 relaxase was observed. This observation has placed Osa DNase activity as a key function of fertility inhibition mechanistic model (**Figure 14**).

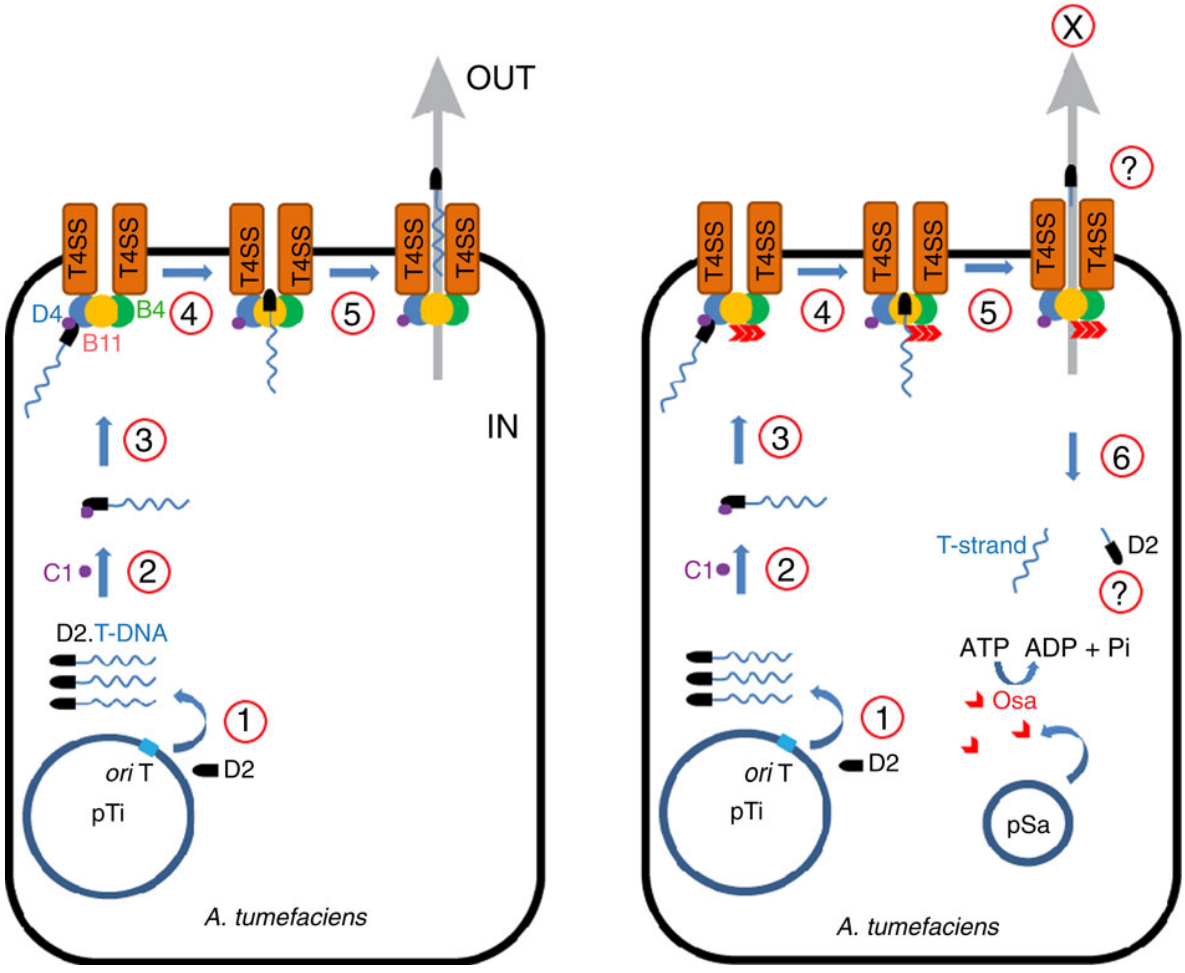



**Figure 14. Mechanistic model of fertility inhibition by Osa.** Left) T-DNA transfer during normal conjugation process. 1) VirD2 relaxase nicks pTi plasmid and binds covalently to T-DNA. 2) VirC1 transfer enhancer adds to T-complex. 3) T-complex docks to the T4CP (VirD4) through the physical interaction VirC1-VirD4. 4) T-DNA covalently bound to VirD2 is located at the cytoplasmic side of the T4SS. 5) T-DNA is channeled through the T4SS pore piloted by VirD2 relaxase. Right) T-DNA possible fate in the presence of an Osa-like fertility inhibition factor. Steps 1, 2, 3, and 4 are the same as in left panel. 5) Osa protein, bound to T4SS through its interaction with VirB4 and VirB11, decouples T-DNA from VirD2 via its DNase activity, blocking T-DNA transfer. 6) Osa protein also modulates VirB4-B11-D4 interaction causing loss of VirD4 affinity to its substrates. Taken from (Maindola *et al.* 2014).

# 4. Artificial barriers to conjugation

After the main barriers that bacteria naturally present to control conjugation have been discussed, attempts to artificially prevent plasmid dissemination will be exposed below. Conjugation inhibitors (COINs) have been employed to target specific components of the conjugation machinery, such as conjugative relaxases or the pilus tip. Other compounds considered COINs inhibited conjugation of several plasmids in different hosts either indirectly, by affecting bacterial growth, or directly by targeting a common conjugative function.

## 4.1. Relaxase inhibitors

Garcillán-Barcia *et al*. obtained stably expressed intracellular antibodies (intrabodies) able to inhibit conjugal transfer of plasmid R388 by blocking relaxase activity in recipient cells (Garcillan-Barcia *et al.* 2007). After mice immunization with TrwC relaxase, immunoglobulin variable sequences were PCR-amplified from mice mRNA, assembled as scFv (single chain variable fragment) antibodies, and cloned into a M13 phagemid vector fused to pIII protein. The resulting library of phagemids was purified and submitted to successive rounds of panning against the purified relaxase immobilized onto ELISA plates, in order to select those antibodies that bind the relaxase more efficiently. The antibody with higher affinity for TrwC was expressed in an *E. coli* strain carrying mutations in the major disulphide bond reduction systems to allow intrabody folding and stability in the reducing cytoplasm of bacteria. It was used as a recipient in a R388 mating experiment, obtaining a 20-fold reduction in transfer frequency compared to a control expressing an unrelated intrabody. To search *in vivo* for more potent intrabodies able to directly inhibit R388 conjugation, a second library from the first round of panning was expressed in recipient cells. The screening was performed by the use of a high-throughput conjugation assay that relies on the emission of luminescence in transconjugant cells (Fernandez-Lopez *et al.* 2005). Several intrabodies inhibited R388 conjugation from 40- to 10,000-fold, confirming that R388 relaxase carried out an important function in recipient cells that could be blocked as a viable strategy to prevent plasmid conjugation. When CloDF13 mobilization by R388 was tested, no change was detected due to the usage of its own relaxase (MobC). An interesting broad range intrabody was discovered that also reduced conjugation of $MOB_F$ plasmids pKM101 and F, whose relaxase domains are 51 and 37 % identical to TrwC, respectively. In addition, mapping the epitopes recognized by one of the intrabodies, the R388 mechanism to terminate conjugative DNA processing was clarified, establishing the second catalytic tyrosine of the relaxase as an important player in this reaction. Although intrabodies are

not the most suitable therapeutic candidates for conjugation control due to the pharmacokinetic problems of any macromolecule as a drug, the recognized epitopes of the relaxase could be targeted by other means in order to generate better applicable COINs.

Relaxase activity of F plasmid TraI was targeted *in vitro* by bisphosphonates, a set of small molecules that could apparently interfere with relaxase active site by mimicking a covalent phosphotyrosine intermediate (Lujan *et al.* 2007). They used a bottom-up approach based on crystallographic data and *in vitro* nicking activity of wt and mutants of TraI relaxase to find compounds that could selectively block its activity. Two of the most potent compounds acting at nanomolar levels were promising hits because they had already been approved to clinically treat bone loss, a fact that would facilitate their inclusion into the market. However, besides the inhibition of F transfer *in vivo*, they also caused unexpected selective death of bacteria containing both a catalytically active TraI and F plasmid. This result was investigated in a second work (Nash *et al.* 2012). Given that bisphosphonates act as metal chelators for osteoporosis treatment, they compared the effect of the chelating agent EDTA and bisphosphonates on donor bacteria survival and conjugative transfer of IncF plasmids F and R100-1. The effect of both compounds was similar, concluding that bisphosphonates may act as mere chelating agents which could affect several other metal-dependent cellular processes.

## 4.2. Pilus blockers

The main inhibitors of bacterial conjugation by blocking conjugative pilus are bacteriophages. Some DNA and RNA bacteriophages use conjugative pilus as receptor to infect bacteria containing certain plasmids. The attachment of these phages to conjugative pili can obstruct potential donor-recipient contacts. This section will focus on COIN activity of male-specific bacteriophages without lytic activity, although phages inducing bacterial lysis are important antimicrobial agents with high potential to fight against AbR bacteria. In fact, the male-specific phage PRD1, with lytic activity against bacteria containing IncP plasmids, was described as an effective plasmid-curing agent. It reduced the frequency of AbR bacteria even under selective pressure for plasmid maintenance, which promoted the emergence of conjugation-deficient mutants resistant to PRD1 (Jalasvuori *et al.* 2011, Ojala *et al.* 2013). Furthermore, lytic phages have caused faster extinction of conjugative plasmids in bacterial populations, probably due to selection for phage resistance mutations that increase genetic burden, indirectly affecting plasmid stability (Harrison *et al.* 2015). Although lytic phages have been amply studied for their use in phage therapy, non-lytic bacteriophages infecting *P. aeruginosa*, such as the filamentous Pf3 and Pf1 phages, could also be effective as antimicrobial

agents thanks to their ability to increase susceptibility to antibiotics (Hagens *et al.* 2006). A similar effect was observed when Boeke *et al*. expressed the pIII protein of F specific filamentous phages, involved in pilus recognition. The presence of pIII in bacterial membranes causes pleiotropic effects, including increased sensitivity to detergents, antibiotics, colicins, and even a reduction in F conjugation and male-specific phage infection, probably by blocking pilus retraction (Boeke *et al.* 1982).

The first report about the COIN activity of bacteriophages originates from an investigation on pilus function. After Brinton *et al*. suggested an association between RNA phage receptors and transport of genetic material (Brinton *et al.* 1964), Knolle found that an inactivated RNA phage fr interfered with F conjugation in the same way that mating partially prevented phage invasion (Knolle 1967). Similar results were obtained with phages f1 and f2 as mating inhibitors, which attach to different sites of F pilus, the tip and the sides, respectively (Ippen and Valentine 1967). F specific DNA and RNA phages (M13 and R17) were employed by Novotny *et al*. to prevent the formation of mating pairs, providing more evidence that support F pilus as the common element involved in an early step of both phage infection and conjugation (Novotny *et al.* 1968). To discard non-specific effects of bacterial growth inhibition caused by phages, transfer of IncF and IncI plasmids present in the same donor cell was blocked by inactivated F and I specific bacteriophages, respectively (Salzman 1971). Using a cell counter to measure mating pairs, Ou demonstrated that f1 and MS2 bacteriophages inhibited MPF completely and partially, respectively. Since the filamentous DNA phage f1 attached to F pilus tip and the RNA bacteriophage MS2 attached laterally along the pilus, the pilus tip was established as the specific attachment site for mating (Ou 1973b). Schreil and Christensen confirmed that MS2 phage interfered with F conjugation, but not due to competition for a common transport channel. Moreover, they disagreed with the reverse effect stated by Knolle (Knolle 1967), noticing that conjugation did not affect MS2 invasion (Schreil and Christensen 1974).

A more recent work (Lin *et al.* 2011) revealed that the inhibition mechanism of F conjugation by M13 involved physical occlusion of conjugative pilus by phage particles (**Figure 15**). Exogenous addition of pIII soluble fragment inhibited conjugation at nanomolar concentrations, whereas addition of the nonspecific protein BSA (bovine serum albumin) did not. This result suggested that the effect was mediated by the phage coat protein pIII, known to interact with the F pilus (Lubkowski *et al.* 1999). The concentration of pIII needed to inhibit F conjugation was 1,000-fold higher than non-replicating phage particles. This apparent higher affinity for F pilus of phage particles than pIII protein alone could be due to the cooperativity between various pIII proteins to bind the pilus, or to the irreversibility of the binding reaction in the case of phage

particles. Lin *et al*. also observed a 5-fold reduction in donor ability when bacteria were infected with replicating phages, probably due to decreased pilus elaboration. This effect could be an important factor at low phage concentrations, when physical occlusion is less important. By constructing a chimeric phage in which the M13 N-terminal domain of pIII was substituted by the homologous sequence of If1 phage, M13 binding specificity was changed from F pilus to I pilus. Consequently, the chimeric phage inhibited conjugation of IncI plasmids instead of F. They also presented a quantitative model for conjugation in the presence of phages that accurately described their COIN effect. Unlike other COINs, bacteriophages have the advantage of potential coevolution in case of resistant bacteria appear.

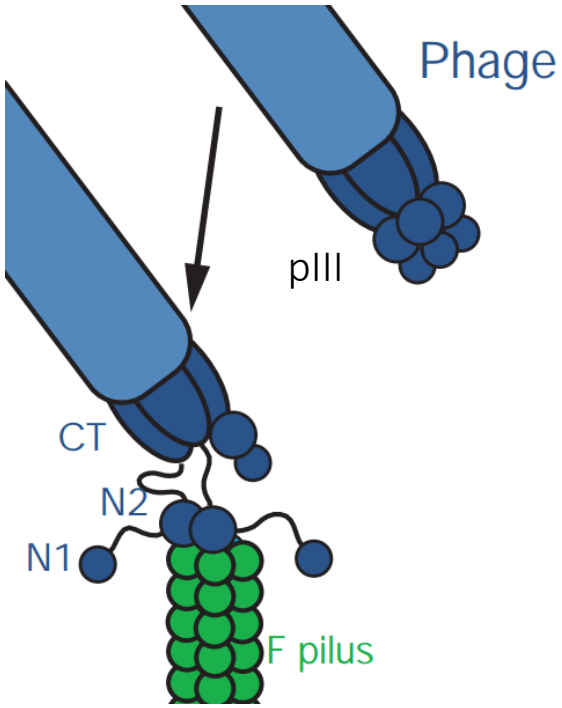



**Figure 15. Inhibition of F conjugation by M13 bacteriophage.** The N2 domain of M13 pIII binds to the F pilus tip as a first step in phage infection, preventing F pilus function during plasmid conjugation. Adapted from (Lubkowski *et al.* 1999).

A kinetic competition study between conjugation and M13 infection suggested that phages must be in significant abundance to be effective antagonists to conjugation. At lower phage concentrations, conjugation persists despite phage inhibition, even in the absence of selective pressure (Wan and Goddard 2012). In spatially structured populations, such as surface-associated colonies and biofilms, the pIII protein of M13 effectively inhibited F conjugation. Moreover, spatial structure itself suppressed F conjugation due to isolation of donor and recipient populations, restricting conjugation to boundaries between them (Freese *et al.* 2014).

Besides conjugation and phage infection, conjugative pili are involved in the elaboration of biofilms, important targets in the battle against resistance (Ghigo 2001). Therefore, bacteriophages affecting F conjugation could also prevent biofilm formation. Actually, male-specific filamentous DNA bacteriophage f1 prevented early biofilm formation by *E. coli* carrying F plasmid. Additionally, the fact that the RNA bacteriophage MS2 did not cause an inhibitory effect suggested that the pilus tip, not the sides, was important for early biofilm formation (May *et al.* 2011).

Antibodies directed against conjugative pilus, able to inhibit conjugation of specific plasmids even more specifically than bacteriophages, were applied to the identification of closely related resistance factors by analyzing their degree of inhibition (Harden and Meynell 1972). These results agreed with previous serological analysis of sex pili detected through antigen-antibody reactions observed by electron microscopy (Lawn and Meynell 1970).

Other described COINs could somehow interfere with elaboration of mating pairs, either by blocking pilus tip in donors, pilus receptor in recipients, or through nonspecific disorganization of bacterial membranes. The case of $Zn^{2+}$ could seem contradictory at the beginning. First, it was found to prevent phage M13 adsorption to F pilus (Tzagoloff and Pratt 1964). Then, a reduction in F donor fertility was shown, probably by blocking pilus tip too, thus inhibiting its interaction with recipient cells (Ou and Anderson 1972). On the contrary, incubation with $Zn^{2+}$ before mating enhanced the ability of recipients to form mating pairs (Ou 1973a). These paradoxical effects were explained by the use of the $Zn^{2+}$ chelator orthophenanthroline (Ou and Reim 1976). $Zn^{2+}$ is probably involved in the formation of receptor sites on the recipient surface and the initial contact could occur between the pilus tip and $Zn^{2+}$ of receptor sites. Therefore, pretreatment of recipient cells increased its fertility through $Zn^{2+}$ incorporation. However, an excess of $Zn^{2+}$ in the mating medium would compete for the tips of F pili, hindering their access to receptor sites. The reduction of $Zn^{2+}$ availability by the mentioned chelating agent drastically decreased conjugation, mainly acting during MPF.

Unlike $Zn^{2+}$, the effect caused by periodate in F donors was irreversible (Sneath and Lederberg 1961). After donor pretreatment, the number of transconjugant cells was greatly reduced, whereas treatment of recipient cells had no significant effect. Perborate and persulfate also decreased donor fertility, but to a lesser extent. The fact that the addition of periodate to a mating in progress did not prevent conjugation of mating pairs already formed suggested an effect on MPF, probably by altering the surface of donor cells via polysaccharide oxidation. Consistent with this observation, the work of Dettori *et al*. showed that periodate also inhibits the adsorption of RNA bacteriophages to the

sides of F pili (Dettori *et al.* 1961). Consequently, an alteration of F pili seems to be the cause of the inhibition of both donor fertility and bacteriophage infection (Ou and Anderson 1972).

The morphine derivative levallorphan, like $Zn^{2+}$ and periodate, inhibited adsorption of the male specific phage MS2 to F pili (Raab and Roschenthaler 1970). This inhibition was comparable to the MPF inhibition during R-factor transfer from *Proteus rettgeri* to *E. coli*. These two effects could be caused by damage of F pilus or the whole bacterial membrane (Loser *et al.* 1971). The tranquillizer chlorpromazine also reduced both IncF plasmid conjugation and adsorption of male specific bacteriophages (Mandi and Molnar 1981). Since this is a cationic amphipathic molecule, it could act by modifying membrane topology through its insertion in the lipid bilayer. Another COIN probably affecting MPF is ammonium (Herrera-Cervera *et al.* 1996). It inhibited conjugation of the rhizobial plasmid pRmeGR4a and pRmeGR4a-assisted mobilization of pRmeGR4b between *Rhizobium meliloti* strains. However, ammonium did not affect conjugation of IncP plasmid RP4 or the rhizobial plasmids to *A. tumefaciens*. Thus, its effect seemed to take place on *R. meliloti* recipient cells, probably on their surface, but not in the transfer machinery.

An inert barrier between donors and recipients is an additional possibility to control bacterial conjugation. Colloidal clay, typically present in natural waters, prevented the transfer of IncF plasmid R1drd19 by forming a coating on bacterial cells (Singleton 1983). This clay envelope was also responsible for *E. coli* protection from bacteriophage infection or predation (Roper and Marshall 1977, Roper and Marshall 1978). In contrast, plasmids that prefer solid surfaces for conjugation, such as IncP plasmid RP4, enhanced their transfer in water containing nanoalumina particles. In the presence of these particles, RP4 upregulated the expression of genes required for MPF (Qiu *et al.* 2012). An unspecified component of *E. coli* cell walls was described as inhibitor of conjugal transfer too. In this case, the inhibitory mechanism presumably involved competence between cell-wall fragments and actual partners on the surface of cells, thereby preventing MPF (Schwartz *et al.* 1965, Lancaster *et al.* 1965).

## 4.3. Nonspecific COINs

Bisphosphonates were first described as relaxase-specific inhibitors (Lujan *et al.* 2007), but then reappraised to act as chelating agents (Nash *et al.* 2012). Similarly, other reported COINs were later revealed as inhibitors of different cellular processes. For instance, the ability of some plasmid-curing agents to inhibit conjugative transfer is easily attributable to their anti-plasmid effect that favors the growth of plasmid-free cells

(Spengler *et al.* 2006). The increased sensitivity of *E. coli* containing F plasmid to bile salts and sodium dodecyl sulfate could be other examples of this effect. While plasmid-free cells were resistant to these toxic detergents, cells with an active system for pilin secretion were more susceptible to their entry through the T4SS pore (Bidlack and Silverman 2004). A similar behavior was found by overexpressing RP4 genes, which caused an enhancement of cell permeability (Daugelavicius *et al.* 1997). Another interesting anti-plasmid effect is mediated by the type VI secretion system (T6SS). T6SSs are used by Gram negative bacteria to kill prokaryotic and eukaryotic cells through contact-dependent delivery of toxic effectors (Russell *et al.* 2014). *P. aeruginosa* T6SS is assembled in response to T6SS attacks by competing bacteria in microbial communities (Basler *et al.* 2013). Besides T6SS, T4SS structural proteins of plasmid RP4 triggered *P. aeruginosa* attacks by T6SS (Ho *et al.* 2013). The work suggested that these donor-directed counterattacks are induced at MPF-mediated membrane perturbations in *P. aeruginosa* recipients to potentially block the acquisition of foreign DNA. Thus, T6SS would represent a new type of immune system against HGT, through a mechanism that indirectly inhibits conjugative transfer by killing donor cells.

Several antimicrobial drugs, even at sub-inhibitory concentrations, have been described as inhibitors of plasmid conjugation. However, their lethal effects in donors and recipients or the absence of COIN activity in non-growing bacteria suggested that these compounds interfere with essential bacterial functions rather than recognizing a specific plasmid target (Weisser and Wiedemann 1987, Debbia *et al.* 1994). In fact, most of these antibiotics act on cellular functions involved in conjugation, such as DNA replication, transcription, translation, or membrane integrity (Viljanen and Boratynski 1991). Similarly, COIN activity of other compounds could be related to their antibacterial activity. This is the case of nitrofurans (Michel-Briand and Laporte 1985) and pipedimic acid (Nakamura *et al.* 1976), which inhibited transfer of several plasmids in different hosts by interfering with DNA replication. Moreover, copper surfaces seem to inhibit conjugal transfer indirectly (Warnes, Highmore*, et al.* 2012), presumably by killing bacteria through DNA and membrane damage (Warnes *et al.* 2010, Warnes, Caves*, et al.* 2012, Hong *et al.* 2012). An antimicrobial component of tea (epigallocatechin gallate) was also described as COIN for plasmid R100 in *E. coli* (Zhao *et al.* 2001). Likewise, carica papaya seed macerate, containing a previously detected antibacterial substance (Emeruwa 1982), was considered a COIN for a *S. typhimurium* conjugative plasmid in the mouse digestive tract at non-lethal concentrations (Leite *et al.* 2005). Another example could be sodium propionate, produced by intestinal bacteria and abundant in the large intestine. It was found to reduce conjugation frequency of IncF plasmid pSLT in the mouse intestine (Garcia-Quintanilla *et al.* 2008). However, it also presented antibacterial properties against several microorganisms (Heseltine and Galloway 1951).

On the contrary, sub-inhibitory concentrations of certain antimicrobial agents can indirectly promote conjugation (Couce and Blazquez 2009). For example, DNA damage caused by ciprofloxacin or mitomycin C induced SOS response, which is responsible for upregulating the excision and transfer of STX ICE from *Vibrio cholerae* (Beaber *et al.* 2004). In a SOS-independent manner, conjugative transposons from *Bacteroides* and *E. faecalis* increased their transfer when exposed to low concentrations of tetracycline (Stevens *et al.* 1993, Torres *et al.* 1991). Similarly, β-lactams stimulated the formation of bacterial aggregates, thus increasing conjugation of a plasmid from *S. aureus* (Barr *et al.* 1986).

In addition to antimicrobial and anti-plasmid agents, COINs affecting many plasmids might be disturbing collateral host processes. Many of them have not been well characterized, usually having unknown targets. An example of a poorly characterized inhibitor was studied in a germ-free mice inoculated with human intestinal flora. Depending on lactose intake and therefore the abundance of the enzymes β-galactosidase and β-glucosidase, R388 plasmid transfer was completely inhibited (lactose intake, both activities increased), reduced (yoghurt and milk intake, β-galactosidase increased and β-glucosidase decreased), or unaffected (standard diet, normal enzymatic levels). These correlated factors were suggested to affect plasmid transfer or maintenance instead of flora composition, moisture, pH, or metabolites (Maisonneuve *et al.* 2002).

## 4.4. Unsaturated fatty acids (uFAs)

The first systematic search for COINs employed plasmid R388 and *E. coli* as a model system (Fernandez-Lopez *et al.* 2005). A luminescence-based high-throughput assay was used to measure R388 conjugation in the presence of more than 12,000 microbial extracts known to contain a wide variety of bioactive compounds. A control assay was run in parallel to discard compounds affecting bacterial growth, plasmid stability, or light emission. The first hits were found during the screening validation of 224 reference chemicals: oleic and linoleic acids, $C_{18}$ fatty acids containing one or two double bonds, respectively. Screening of the NatChem library provided 48 active extracts, which were subjected to fractionation and a second round of search. The 52 most active fractions were analyzed by conventional conjugation assays using IncW, IncF, and IncP plasmids. Most active fractions against R388 and R1 contained linoleic acid. An atypical fatty acid named dehydrocrepenynic acid (DHCA) was identified in a fraction obtained from the fungus *Sistotrema sernanderi*. DHCA is a $C_{18}$ fatty acid with double bonds at positions 9 and 14, and a triple bond at position 12. Conjugation analysis using saturated fatty acids and related compounds suggested that carboxylic group, long carbon chain, and double

bond position were important features of COINs. While $MIC_{98}$ values of oleic and linoleic acids were about 400 μM, DHCA $MIC_{98}$ was 70 μM. Affected plasmids by linoleic acid and DHCA were R388 and pOX38, whereas RP4 or R6K were not. The fact that some plasmids were not affected could discard general metabolic disturbances as cause. In addition, these results suggested that the inhibition target was involved in DNA processing (MOB), more similar between R388 and pOX38 than RP4 and R6K (Francia *et al.* 2004). Accordingly, CloDF13 mobilization by R388 MPF was not affected, reinforcing this hypothesis. However, the absence of effect on IncN plasmid pKM101, with a MOB module more similar to R388 and pOX38 than to RP4, weakened the hypothesis. Moreover, the ATPase activity of the R388 MPF component TrwD was inhibited by uFAs (Ripoll-Rozada *et al.* 2016). TrwD ATPase activity is involved in pilus biogenesis and DNA translocation (Kerr and Christie 2010, Atmakuri *et al.* 2004), so its inhibition would block bacterial conjugation. TrwD interacts with the bacterial inner membrane (Machon *et al.* 2002). Significantly, other proteins associated with bacterial membranes are inhibited by uFAs, some of them also being ATPases (Swarts *et al.* 1990, Yung and Kornberg 1988, Mahmmoud and Christensen 2011, Haag *et al.* 1999). Nevertheless, the absence of known TrwD homologs in IncF plasmids (Guglielmini *et al.* 2014) still leaves unanswered questions. The target in these plasmids, well adapted to *E. coli* after a long history of co-evolution, could be a chromosomal ATPase or another plasmid ATPase, such as the TrwK-homolog TraC, the only ATPase of plasmid F essential for pilus biogenesis (Firth *et al.* 1996).

### 4.5. Potential new COINs

Novel approaches could be employed in the near future to discover more COINs, taking advantage of already generated knowledge. A portion of this knowledge could be the identification of dimerization inhibitors of the T4SS component VirB8 from *Brucella abortus* (Paschos *et al.* 2011, Smith *et al.* 2012). Although they were discovered as virulence inhibitors of this intracellular pathogen, their inhibitory effect could be extrapolated to VirB8 homologs of conjugation systems. In fact, IncP RP4 and IncN pKM101 conjugative plasmids were analyzed in the presence of the most potent VirB8 inhibitors (B8I-1 and B8I-5), but they showed no effect at concentrations up to 50 μM (Paschos *et al.* 2011). Nevertheless, structural information about their mechanism of action might be useful for future development of VirB8 inhibitors in conjugative T4SSs.

VirB11 ATPase is another component of the T4SS that has been subject to inhibitors development, with the aim of preventing *Helicobacter pylori* virulence. The first described inhibitors of a T4SS targeted the *H. pylori* VirB11-type ATPase Cagα, blocking CagA toxin transport to host cells. The most active compound (CHIR-1, identified by a

high-throughput screening that measured ATPase activity in the presence of small compounds libraries) reduced *H. pylori* pathogenic effects in gastric cells and the ability of treated bacteria to colonize gastric mucosa in mice (Hilleringmann *et al.* 2006). In a recent study, docking analysis using CagΑ allowed the identification of a series of competitive inhibitors with potential as antibacterial agents (Sayer *et al.* 2014). These anti-virulence compounds could be tested in conjugative VirB11 homologs, such as TrwD of IncW plasmid R388.

Type III secretion systems (T3SSs) of several pathogens were also targeted by new anti-virulence drugs. They generally act as needles to inject virulence effectors into host cells (Galan *et al.* 2014). Several compounds were found to block T3SS in different pathogenic bacteria (Charro and Mota 2015). An interesting work developed a whole-cell high-throughput screening of T3SS inhibitors based in *Salmonella typhimurium*. A compound was identified that inhibited both T3SS and T2SS, probably by targeting an outer membrane component conserved between these two secretion systems (Felise *et al.* 2008). These results provide a proof of concept that compounds with a broad spectrum of activity against different bacterial secretion systems could be developed.

# Aims and scope

The main purpose of this work was to find new mechanisms to control bacterial conjugation, both natural and artificial, which could help to prevent the dissemination of antibiotic resistance determinants.

The specific aims were:

1. To search for *E. coli* functions in donor and recipient bacteria involved in conjugation of the broad host range plasmid R388.
2. To study the interactions between unrelated conjugative plasmids in donor bacteria, in order to find new fertility inhibition systems.
3. To look for natural and synthetic compounds that act as specific conjugation inhibitors (COINs).
4. To investigate the chemical characteristics important for COIN activity, as well as the effect of COINs in different plasmids, hosts, and bacterial populations.

# Publications

# 1. *Escherichia coli* functions limiting plasmid conjugation

**Getino M**, Palencia-Gándara C, Campos-Gómez J, del Campo I, de la Cruz F. Manuscript in preparation.

## 1.1. Abstract

Bacterial conjugation is the main mechanism causing the dissemination of antibiotic resistance genes. Therefore, searching for targets and barriers to inhibit conjugation could unveil effective new strategies to control antibiotic resistance dissemination. The conjugative plasmid R388, able to propagate between distantly related bacteria, was used as test plasmid for a high-throughput screening of host genes involved in conjugation. In a previous work, no non-essential genes were found in *E. coli* recipient cells essential for R388 entry. This work identified *E. coli* genes whose deletion in donor cells affected R388 conjugation efficiency. Results indicated that deletion of some functions related with bacterial membrane composition and ATP synthesis inhibited R388 conjugation up to 10-fold, possibly by affecting either mating pair formation or the function of any of the four conjugative ATPases. In addition, no mutants significantly increased conjugation, either as donor or recipient cells, suggesting that no specific gene in *E. coli* DH5α lab strain results in a barrier for conjugation. On the contrary, when using a collection of clinically isolated enterobacteria as recipients in similar conjugation experiments, 80 % of them blocked R388 conjugation. Two different mechanisms of inhibition were detected. On the one hand, bacteriocin production by colicinogenic plasmids efficiently killed donor cells. On the other hand, restriction-modification systems abolished DNA acquisition through either conjugation or transformation. Overall, our results suggest that plasmid conjugation in *E. coli* is controlled by strain-specific mechanisms, which act on a background of conjugation-permissive genetic makeup.

## 1.2. Introduction

Bacterial conjugation allows the dissemination of diverse adaptive traits between distantly related bacteria, reaching human pathogens (Amabile-Cuevas and Chicurel 1992). Specifically, antibiotic resistance (AbR) genes are distributed in the environment mainly by conjugative plasmids (Waters 1999, Norman *et al.* 2009). AbR pathogens cost many human lives and resources annually (Cooper and Shlaes 2011). The overuse of antibiotics and the adaptability of bacteria are important negative factors contributing to this problem, difficult to address at global scale (Davies and Davies 2010).

Consequently, understanding bacterial conjugation through searching for barriers, targets or, in general, mechanisms involved in conjugation, seems key to find potential solutions.

Bacterial defenses have naturally evolved to fight potentially harmful incoming DNA from bacteriophages or conjugative plasmids, using mechanisms such as restriction-modification (RM) (Wilkins 2002) or CRISPR-Cas systems (Marraffini and Sontheimer 2008). In this sense, few works have systematically looked for genes relevant to the conjugation process, as a means to distinguish among the different mechanisms to control the dissemination of the great diversity of conjugative plasmids. In *Escherichia coli*, a collection of mutants was screened as recipients of IncW plasmid R388 (Perez-Mendoza and de la Cruz 2009). No non-essential genes were found to be substantially required for conjugation. However, mutations in genes involved in lipopolysaccharide (LPS) biosynthesis, with a modest effect in R388 surface mating, had a drastic effect in F liquid mating, enhancing the importance of recipient LPS in mating pair stabilization. Accordingly, ICE*Bs1* conjugation was affected when *Bacillus subtilis* deficient in functions associated with membrane composition were used as recipients (Johnson and Grossman 2014).

Since conjugation could not be possible without donor bacteria, the Keio collection of *E. coli* deletion mutants (Baba *et al.* 2006) was tested as donors of R388, a broad host range (BHR) plasmid interchanged between distantly related bacteria (Mazodier and Davies 1991). In addition, a collection of clinically isolated enterobacteria was analyzed as recipients of R388 to test the ability of different pathogenic bacteria to accept conjugative plasmids. Hence, the purpose of this work was to find new potential functions in donor or recipient bacteria important for plasmid dissemination.

## 1.3. Results

### Keio mutants act as capable donors for R388 conjugation

A high-throughput conjugation (HTC) screening based on the emission of fluorescence in transconjugant bacteria was employed to search for donor functions affecting plasmid conjugation on solid media. The test plasmid used was a derivative of IncW plasmid R388 (pJC01) expressing a fluorescent protein when it enters recipient cells, that is, when T7 polymerase activates *gfp* promoter (Getino *et al.* 2015). Thus, the test plasmid was introduced by conjugation into each of the 3,908 mutants of the Keio collection, and then each mutant was used as donor of the test plasmid. Plasmid pJC01 conjugation was detected in recipient bacteria through fluorescence measurements after

mating. At least two independent conjugation frequency (CF) values, estimated as the ratio GFP/$OD_{600}$ and normalized to the mean CF of the corresponding plate, were obtained for each of the mutants. The mean of the normalized CF obtained for each mutant was represented in **Fig 1**.

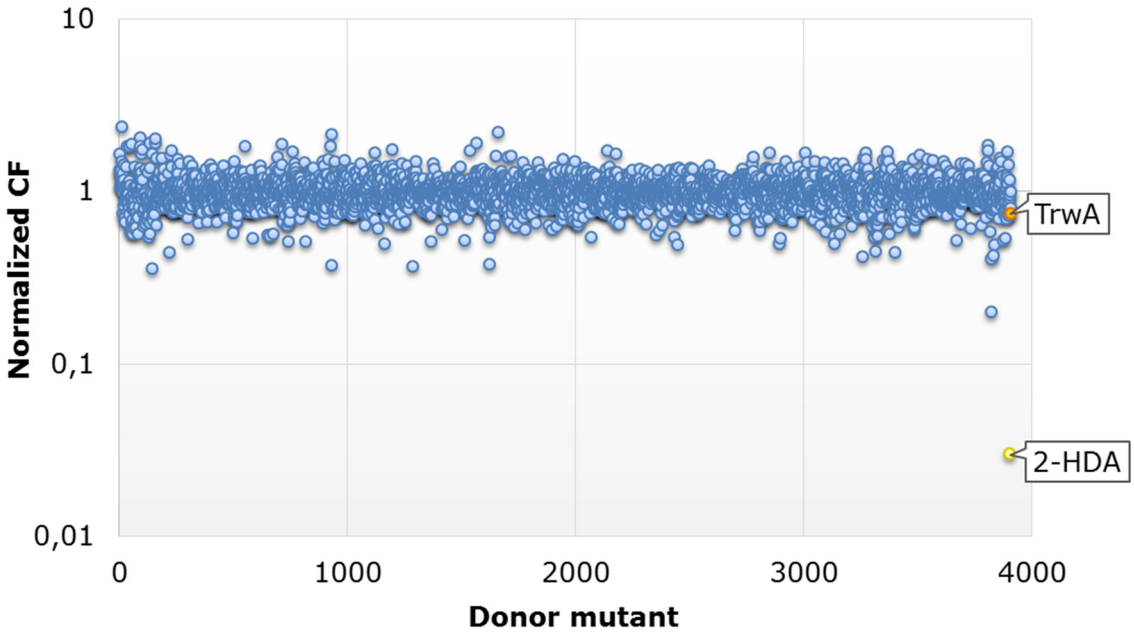



**Fig 1. HTC screening of Keio mutants as donor bacteria.** Mean of at least two CF values obtained for each deletion mutant using the fluorescence-based HTC assay (blue circles), normalized to the mean CF of the corresponding plate. TrwA (orange), control of reduced donor ability by overexpressing TrwA regulator (pSU1545) (Moncalian *et al.* 1997) in donor *E. coli* MDS42 (pJC01), normalized to the mean CF obtained with the empty vector pKK223-3 instead of pSU1545. 2-HDA (yellow), control of reduced donor ability by adding 2-HDA 0.3 mM to the mating medium (Getino *et al.* 2015), using *E. coli* DH5α (pJC01) as donor.

To complete the screening, a set of 69 corrected mutants substituted the incorrect mutants detected in the Keio collection (Yamamoto *et al.* 2009) and the results obtained were included in **Fig 1**. Additionally, a set of 25 verified undisrupted strains, obtained when deletion of potentially essential genes was attempted, were used as controls of normal donor ability. In fact, 14 of these genes were reported to be essential for growth in PEC database (http://www.shigen.nig.ac.jp/ecoli/pec). As observed in **Fig 2**, these undisrupted strains showed relative CF ranging from 0.8 to 1.3, thus being considered ideal controls as wild type (wt) donors containing the kanamycin cassette (unlike the Keio parental strain BW25113). Among them, the undisrupted strain of the essential DNA polymerase I (*polA* gene) (Joyce and Grindley 1984, Olivera and Bonhoeffer 1974),

with relative CF of 1.1 ± 0.2 (**Fig 2**), was selected as positive control for plate-conjugation assays to confirm the effects observed in the primary HTC assay.

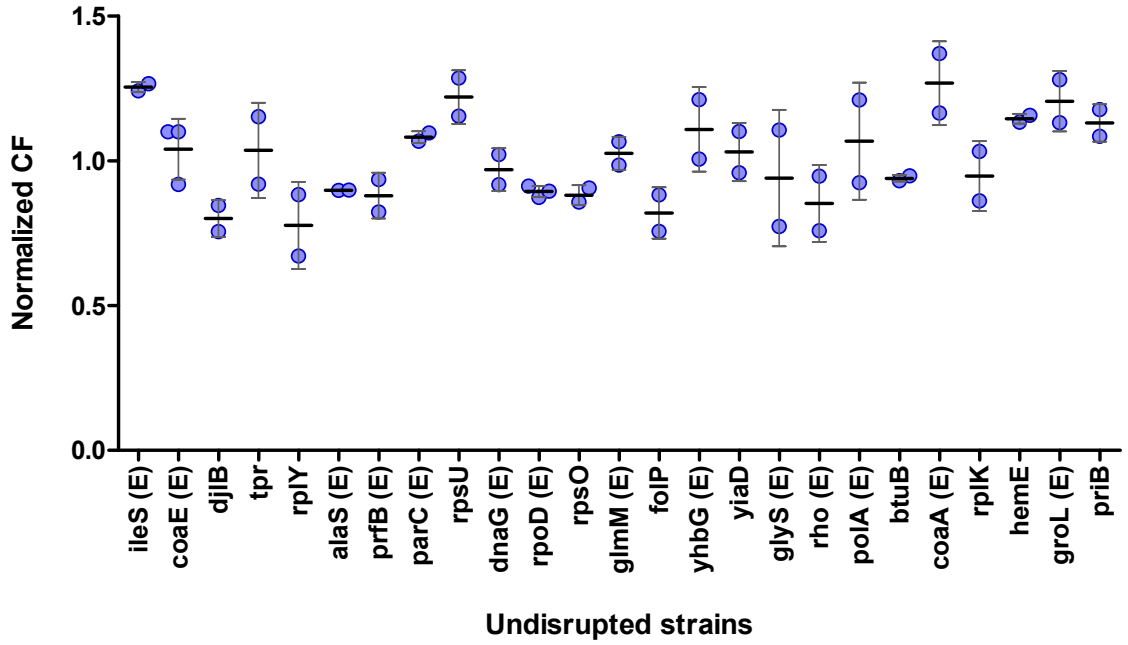



**Fig 2. Undisrupted strains as control donors in HTC assay.** CF obtained by each undisrupted strain as donors in the primary fluorescence-based HTC assay, normalized to the mean CF of the corresponding plate. Each point represents the result of one experiment. Horizontal and vertical bars represent the mean ± SD of at least two experiments. (E), genes essential for *E. coli* growth according to PEC database.

No drastic effect was found when using any of the Keio deletion mutants as donor bacteria. In fact, only 10 mutants gave CF under 0.5 and only 5 mutants over 2.0 (**Fig 1**). Although ascertain potential targets or barriers to conjugation seems difficult with so modest effects, ranging from half to double, the 20 mutants with the most extreme CF were re-assayed to confirm hits as well as to discard those mutations affecting factors from the HTC assay, such as GFP emission or long conjugation times.

First, the 10 donor mutants with CF under 0.5 in the primary HTC assay were analyzed by plate-conjugation assay. Results shown in **Table 1** confirmed the significant contribution of 7 mutations to low CF, ranging from 0.07 to 0.28, after normalizing to the mean CF of the positive control (*polA*$^-$ undisrupted strain as donor). As control of reduced donor ability, the R388 regulator TrwA was overexpressed in donor cells. The mean CF normalized to the control in the absence of TrwA was 0.17 ($p < 0.001$). The mutant that most affected R388 conjugation was *mdtA*$^-$, a gene encoding a component of a multidrug efflux system involved in AbR. Interestingly, other proteins associated

with bacterial membrane (such as RssA, YcaL, or YdfJ) also seemed to be directly or indirectly involved in conjugation, since their deletion in donors lowered R388 transfer up to 10 times (**Table 1**). In addition, *atpB*$^-$ and *atpD*$^-$ mutants affecting ATP synthesis significantly diminished conjugation. To complement these results, *atpA*$^-$ and *atpE*$^-$ mutants were also tested as donors deficient in ATP synthase function. They showed CF of 0.5 ± 0.2 and 0.6 ± 0.3, respectively, in the primary HTC assay. As occurred with *atpB*$^-$ and *atpD*$^-$ (**Table 1**), *atpA*$^-$ and *atpE*$^-$ mutants showed significant reductions in plate-conjugation assays (0.19 and 0.16 with $p < 0.001$, respectively).

**Table 1. Donor mutants diminishing R388 conjugation.**

| Mutant | HTC | PC | Function |
|---|---|---|---|
| ***atpB***$^-$ | 0.2 ± 0.1 | 0.25 ** | F0 sector of membrane-bound ATP synthase, a subunit: ATP-proton motive force interconversion |
| ***fumC***$^-$ | 0.4 ± 0.1 | 0.13 ** | Fumarate hydratase class II: carbon catabolism |
| ***rssA***$^-$ | 0.4 ± 0.1 | 0.21 *** | Putative patatin-like family phospholipase |
| ***ycaL***$^-$ | 0.4 ± 0.2 | 0.28 *** | Putative metalloprotease (lipoprotein) |
| ***hisD***$^-$ | 0.4 ± 0.2 | 0.78 | Histidinol dehydrogenase: histidine biosynthesis |
| ***atpG***$^-$ | 0.4 ± 0.4 | 0.63 | F1 sector of membrane-bound ATP synthase, γ subunit: ATP-proton motive force interconversion |
| ***mdtA***$^-$ | 0.4 ± 0.2 | 0.07 *** | Multidrug efflux system, subunit A |
| ***atpD***$^-$ | 0.4 ± 0.4 | 0.36 * | F1 sector of membrane-bound ATP synthase, β subunit: ATP-proton motive force interconversion |
| ***ydfJ***$^-$ | 0.4 ± 0.2 | 0.11 *** | Putative inner membrane metabolite transport protein |
| ***hrpA***$^-$ | 0.4 ± 0.1 | 0.54 | Putative ATP-dependent RNA helicase: mRNA processing |

Relative CF confirmed by plate-conjugation assay (PC) of the ten mutants with the lowest CF obtained in the primary fluorescence-based HTC assay (HTC, mean ± SD). CF (PC) represents the mean of at least eight experiments, calculated as the ratio of transconjugant cells per donor and normalized to the mean of the positive control (*polA*$^-$ undisrupted strain as donor). The recipient strain used was *E. coli* MDS42 and the initial donor-recipient ratio was 1:10. Mean significantly different from control with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The function of the gene deleted in each mutant was included in the table (source: http://www.ecogene.org).

### No donor or recipient mutants significantly improve R388 conjugation

Other 10 Keio mutants were re-assayed as donors increasing R388 conjugation more than 1.8-fold in HTC assay. In this case, no mutants significantly increased conjugation. Instead, two mutants with deletions in a repressor of replication initiation (*seqA*) and an enzyme involved in carbon catabolism (*sucA*) decreased conjugation 10-fold (**Table 2**).

**Table 2. Donor mutants increasing R388 conjugation.**

| Mutant | HTC | PC | Function |
|---|---|---|---|
| ***aceF*** $^{-}$ | 2.4 ± 0.4 | 1.33 | Pyruvate dehydrogenase, dihydrolipoyltransacetylase component E2: acetyl-CoA biosynthesis |
| ***cysE*** $^{-}$ | 2.2 ± 1.5 | 1.74 | Serine acetyltransferase: cysteine biosynthesis |
| ***yafE*** $^{-}$ | 2.2 ± 0.4 | 1.32 | Putative S-adenosyl-L-methionine-dependent methyltransferase |
| ***nuoL*** $^{-}$ | 2.1 ± 0.3 | 0.68 | NADH:ubiquinone oxidoreductase subunit L, complex I: aerobic respiration |
| ***nuoF*** $^{-}$ | 2.0 ± 0.1 | 0.56 | NADH:ubiquinone oxidoreductase subunit F, complex I: aerobic respiration |
| ***nuoN*** $^{-}$ | 1.9 ± 0.6 | 0.80 | NADH:ubiquinone oxidoreductase subunit N, complex I: aerobic respiration |
| ***nuoH*** $^{-}$ | 1.9 ± 0.0 | 0.54 | NADH:ubiquinone oxidoreductase subunit H, complex I: aerobic respiration |
| ***seqA*** $^{-}$ | 1.9 ± 0.5 | 0.11 *** | Negative modulator of initiation of replication |
| ***ybeY*** $^{-}$ | 1.9 ± 0.4 | 0.97 | ssRNA-specific endoribonuclease, 16S rRNA 3' end maturation and quality control co-endoribonuclease working with RNase R, rRNA transcription antitermination factor |
| ***sucA*** $^{-}$ | 1.9 ± 0.3 | 0.15 *** | 2-oxoglutarate dehydrogenase, E1 component: carbon catabolism |

Relative CF confirmed by plate-conjugation assay (PC) of the ten mutants with the highest CF obtained in the primary fluorescence-based HTC assay (HTC, mean ± SD). CF (PC) represents the mean of at least six experiments, calculated as the ratio of transconjugant cells per donor and normalized to the mean of the positive control (*polA*$^{-}$ undisrupted strain as donor). The recipient strain used was *E. coli* MDS42 and the initial donor-recipient ratio was 1:10. Mean significantly different from control with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The function of the gene deleted in each mutant was included in the table (source: http://www.ecogene.org).

Since the previous study using mutations in recipient bacteria was focused on conjugation inhibitory effects (Perez-Mendoza and de la Cruz 2009), the 40 Keio mutants with higher CF were re-tested by the luminescence-based HTC assay described in that work. As a result, 10 recipient mutants with relative CF over 1.5 were selected for further analysis. However, none of them produced CF significantly higher than *polA*$^{-}$ undisrupted strain as recipient (**Table 3**).

**Table 3. Recipient mutants increasing R388 conjugation.**

| Gene | HTC | PC | Function |
|---|---|---|---|
| ***ybiX*⁻** | 1.9 ± 0.2 | 1.9 | Fe(II)-dependent oxygenase superfamily protein |
| ***rnr*⁻** | 1.8 ± 0.3 | 1.1 | Exoribonuclease R: RNA maturation and stability |
| ***ychF*⁻** | 1.8 ± 0.2 | 1.9 | Catalase inhibitor protein, ribosome-binding $K^+$-dependent ATPase |
| ***torR*⁻** | 1.8 ± 0.2 | 1.5 | Response regulator in two-component regulatory system with TorS: anaerobic utilization of trimethylamine-N-oxide |
| ***yciU*⁻** | 1.8 ± 0.2 | 1.9 | UPF0263 family protein |
| ***yceA*⁻** | 1.7 ± 0.2 | 2.1 | Putative rhodanese-related sulfurtransferase: cyanide detoxification |
| ***fis*⁻** | 1.6 ± 0.2 | 1.4 | Global DNA-binding transcriptional dual regulator |
| ***mdoH*⁻** | 1.6 ± 0.1 | 1.9 | ACP-dependent transmembrane UDP-glucose beta-1,2 glycosyltransferase, nutrient-dependent cell size regulator, FtsZ assembly antagonist: osmoregulated periplasmic glucan biosynthesis |
| ***ymcC*⁻** | 1.6 ± 0.1 | 2.0 | Predicted outer membrane lipoprotein required for formation of the O-antigen capsule: LPS biosynthesis |
| ***cusS*⁻** | 1.6 ± 0.2 | 1.8 | Copper-sensing histidine kinase in two-component regulatory system with CusR |

Relative CF confirmed by plate-conjugation assay (PC) of the ten mutants with highest CF obtained in the luminescence-based HTC assay. CF (HTC) shows the mean ± SD of four experiments normalized to the positive control (*E. coli* BW25113 as recipient). CF (PC) represents the mean of at least six experiments, calculated as the ratio of transconjugant cells per recipient and normalized to the mean of the positive control (*polA*⁻ undisrupted strain as recipient). The donor strain used was *E. coli* MDS42 (pJC01) and the initial donor-recipient ratio was 1:10. Mean significantly different from control with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The function of the gene deleted in each mutant was included in the table (source: http://www.ecogene.org).

### Most clinically isolated enterobacteria inhibit R388 conjugation

*E. coli* lab strains are good recipients for conjugative plasmids (Perez-Mendoza and de la Cruz 2009). On the contrary, wt *E. coli* strains might encode defense barriers to conjugation, such as RM or CRISPR-Cas systems (Wilkins 2002, Marraffini and Sontheimer 2008). They could also contain plasmids with incompatible replication or exclusion systems affecting conjugation of other plasmids (Taylor *et al.* 2004, Garcillan-Barcia and de la Cruz 2008). In order to analyze the ability of wt clinical isolates to affect conjugation, a set of 117 multi-resistant enterobacteria was tested as recipients using a HTC assay based on the emission of luminescence in transconjugant cells (Perez-Mendoza and de la Cruz 2009). The resulting luminescence values were normalized to the positive control using *E. coli* DH5α as recipient strain (100 %). Out of 117 wt strains

used as recipients, 79 % showed CF under 10 % (**Fig 3A**). To find out the influence of resident plasmids in conjugation control by wt recipients, a collection of 199 lab strains carrying conjugative or mobilizable plasmids obtained from clinical isolates (transconjugants) were analyzed. In this case, only 39 % of them inhibited conjugation more than 10 % (**Fig 3B**), suggesting that most control mechanisms were encoded by the bacterial chromosome of clinical enterobacteria.

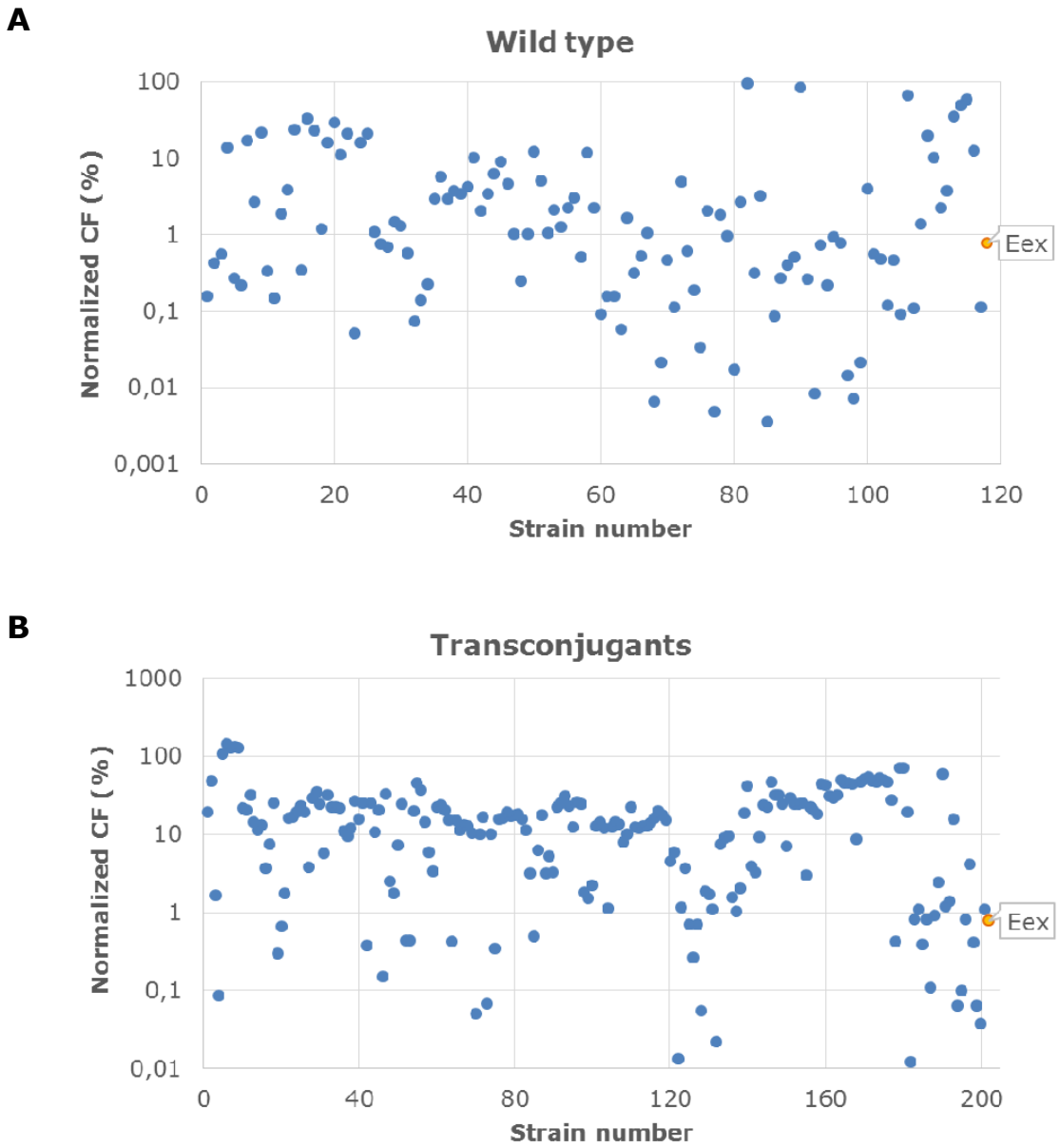



**Fig 3. HTC screening of clinical isolates as recipient bacteria of plasmid R388.** Mean of at least six CF values obtained for each recipient (wt in panel A and transconjugants in panel B). Results were obtained using the luminescence-based HTC assay and normalized to the mean CF of the positive control (*E. coli* DH5α as recipient, 100 %). Eex (orange), control of reduced recipient ability by overexpressing R388 entry exclusion (pSU5024) in recipient *E. coli* DH5α (Fernandez-Lopez *et al.* 2005).

**Plasmids of recipient bacteria produce bacteriocins against donors**

In order to find novel mechanisms of inhibition encoded by plasmids, *E. coli* DH5α transconjugants (Alvarado 2016) inhibiting R388 conjugation by more than 100-fold were selected for further analysis (**Table 4**).

**Table 4. Recipients reducing R388 conjugation.**

| Transconjugant | CF (%) | Wt | MOB |
|---|---|---|---|
| 11003 | 0.42 | 3315 | F12, P3 |
| 11084 | 0.01 | 3632 | F12, P51 |
| 11100 | 0.82 | 3062 | P12, P51 |
| 12033 | 0.11 | 3942 | C12, P12, P51 |
| 13022 | 0.06 | 3899 | C11, C12, P12, P3, P51 |
| 13091 | 0.06 | 3989 | P51, qu |
| 13101 | 0.04 | 3271 | P3, P51 |

CF obtained in the primary luminescence-based HTC assay for each selected recipient, normalized to the mean CF of the positive control (*E. coli* DH5α as recipient, 100 %). MOB, MOB type of plasmids detected in *E. coli* wt strains from urinary tract infections isolated in Umeå hospital (Ejrnaes *et al.* 2011, Alvarado *et al.* 2012, Alvarado 2016).

Since no $MOB_{F11}$/IncW plasmids were detected in any of the original isolates (**Table 4**), incompatibility or exclusion systems carried by R388-like plasmids were unlikely to be the cause of R388 inhibition of conjugation. Instead, most of the selected DH5α transconjugants killed donors and, consequently, reduced conjugation. As observed in **Fig 4**, 6 out of 7 selected recipients produced an inhibitory halo when their filtered supernatants were spotted on a plate spread with *E. coli* DH5α test strain. In addition, these haloes presented variable diameter sizes, suggesting that the antimicrobial molecules produced had different diffusion abilities. Interestingly, the recipients capable of killing DH5α cells derived from strains with $MOB_{P51}$ and $MOB_{C11}$ relaxases (**Table 4**), typically present in colicinogenic plasmids such as ColE1 and CloDF13 (Garcillan-Barcia *et al.* 2009). Recipient 11003 was excluded for subsequent analysis due to its multiple AbR.

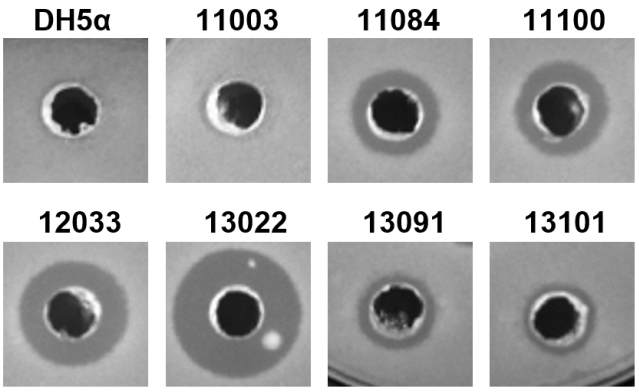



**Fig 4. Growth inhibition test by selected recipients.** Photograph of a plate cultured with *E. coli* DH5α test strain in which filtered supernatant cultures of the named recipients were spotted. Inhibition haloes represent the production of antimicrobial molecules by the selected recipients. DH5α, supernatant of a non-producer strain. Inhibition zone diameters (mm): 7 (DH5α), 7 (11003), 10 (11084), 11 (11100), 12 (12033), 15 (13022), 9 (13091), 8 (13101).

### Pathogenic *E. coli* prevents horizontal gene transfer through RM systems

Isolate 3065 was selected as an example of a clinical *E. coli* strain isolated from urinary tract infections (Ejrnaes *et al.* 2011) that blocks the entry of R388 plasmid (relative CF of 1.8 % in the primary HTC assay). An ApR $MOB_{P12}$ plasmid was detected (Alvarado *et al.* 2012, Alvarado 2016) and transferred to an empty lab strain to check its ability to inhibit R388 conjugation in the recipient. Plate-conjugation assays were performed to discard any factor affecting the primary HTC assay, such as light emission or bacterial growth. As a result, the original strain 3065 reduced R388 conjugation more than 6-fold, whereas the ApR plasmid in the *E. coli* lab strain CSH53 did not affect the entry of the plasmid, discarding the plasmid as the inhibition source (**Fig 5**). As control of reduced recipient ability, R388 entry exclusion (Eex) overexpressed in recipient *E. coli* DH5α reduced R388 conjugation to 0.2 %, relative to the control in the absence of Eex (**Fig 5**).

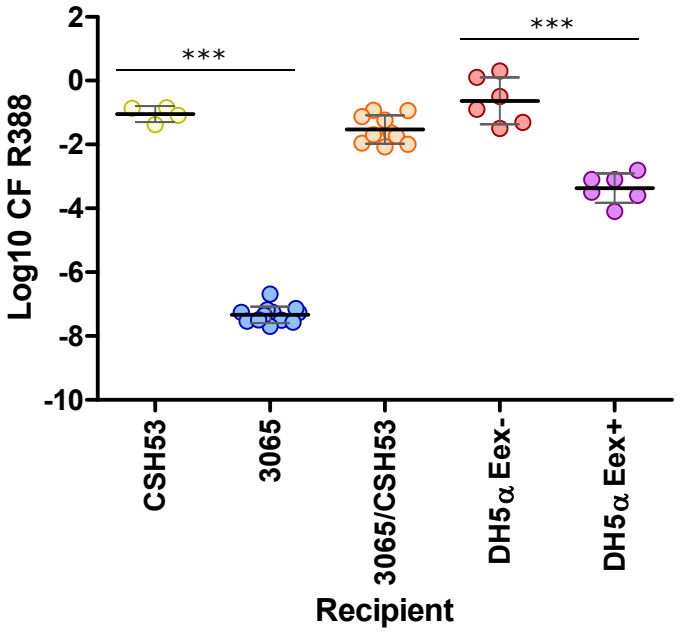



**Fig 5. R388 conjugation to recipient 3065.** CF of plasmid R388 using *E. coli* CSH53 (yellow), 3065 (blue), or CSH53 containing the ApR plasmid from 3065 (orange) as recipient strains. DH5α $Eex^+$ (purple), control of reduced recipient ability by overexpressing R388 entry exclusion (pSU5024) in recipient bacteria (Fernandez-Lopez *et al.* 2005). DH5α $Eex^-$ (red), control in the absence of Eex (empty pET3a) in recipient bacteria. Each point shows the result of one experiment measured by plate-conjugation assay in logarithmic scale, calculated as the ratio of transconjugant cells per recipient. Horizontal and vertical bars represent the mean ± SD for each group of data. Mean significantly different from the corresponding control with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The CF values obtained when 3065 was used as recipient were lower than those displayed in the figure.

Besides R388 (IncW), three other conjugative plasmids clinically representative from enterobacteria were tested, using the control strain DH5α or the clinical isolate 3065 as recipients. These plasmids were pOX38 (IncFI), R100-1 (IncFII), and R751 (IncP1). The results obtained (**Fig 6**) showed CF at least 6-log lower than the control strain (DH5α) using R388, R100-1, or R751 as conjugative plasmids, and a 4-log reduction when pOX38 was employed. These results suggest that the inhibitory mechanism, presumably encoded by 3065 chromosome, blocked the entry of a broad diversity of conjugative plasmids.

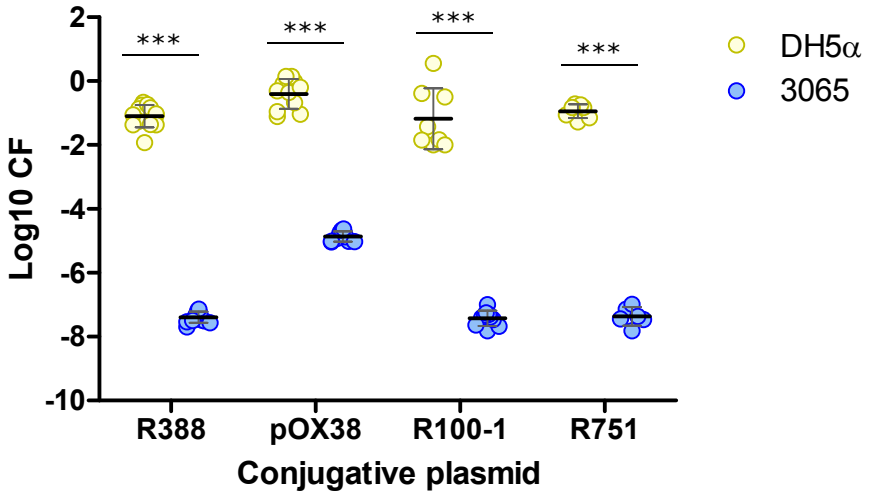



**Fig 6. Plasmid conjugation to recipients DH5α and 3065.** CF of four clinically representative plasmids using DH5α or 3065 as recipient strains (yellow or blue, respectively). Each point shows the result of one experiment measured by plate-conjugation assay in logarithmic scale, calculated as the ratio of transconjugant cells per recipient. Horizontal and vertical bars represent the mean ± SD for each group of data. Mean significantly different from the corresponding control with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The CF values obtained for plasmids R388, R100-1, and R751 using 3065 as recipient were lower than those displayed in the figure.

Although plasmids can carry RM systems as stabilization mechanism (Kobayashi 2001), only 10.5 % of them encode these systems (Oliveira *et al.* 2014). On the other hand, most of the prokaryotic chromosomes (69 %) present RM systems, mainly as innate defense against invading genomes (Oliveira *et al.* 2014). Since conjugation of four different plasmids was found to be blocked by 3065 recipient (**Fig 6**), other types of horizontal gene transfer, such as transformation, could also be inhibited. Three different plasmids were transformed to test whether RM systems were responsible for 3065 inhibitory action. *E. coli* DH5α (control strain) or 3065 were used as recipients of plasmids pSU19 (~ 2 kb), ColE1::*KmR* (~ 7 kb), or RSF1010::*KmR* (~ 9 kb). When those plasmids were extracted from DH5α, 3065 transformation was similar to control with the smallest plasmid, 2-log lower than the control with the medium-size plasmid, and more than 4-log lower with the largest plasmid (**Fig 7**). In addition, when the plasmids ColE1::*KmR* and RSF1010::*KmR* were extracted from 3065 (presumably capable of modifying its own DNA to protect it from RM endonucleases), their ability to be transformed into 3065 was restored (**Fig 7**).

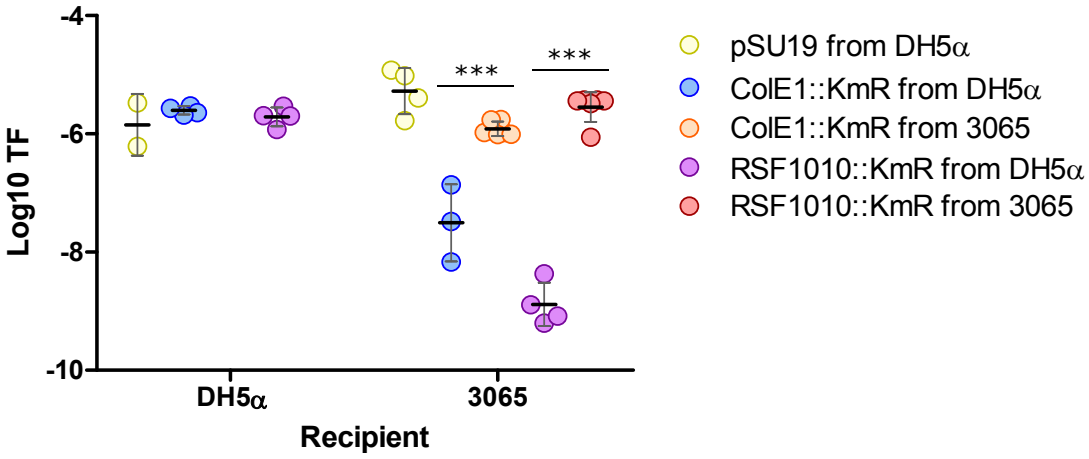



**Fig 7. Transformation ability of recipient 3065.** Transformation frequency of *E. coli* DH5α or 3065 with plasmids pSU19 (~ 2 kb) (Bartolome *et al.* 1991), ColE1::*KmR* (~ 7 kb) (Bodsch 1977), or RSF1010::*KmR* (~ 9 kb) (Butler and Gotschlich 1991) extracted from *E. coli* DH5α or 3065. Each point shows the result of one experiment in logarithmic scale. Horizontal and vertical bars represent the mean ± SD for each group of data. Mean significantly different from the corresponding control with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The values obtained when 3065 was transformed with plasmid RSF1010::*KmR* extracted from DH5α (purple) were lower than those displayed in the figure.

## 1.4. Discussion

Unlike narrow host range plasmids, which rely on a series of host-encoded regulatory factors for conjugation control (Frost and Koraimann 2010), BHR plasmids regulate their transfer mainly through plasmid-encoded repressors (Fernandez-Lopez *et al.* 2014). Since they can be transferred between distantly related bacteria (Mazodier and Davies 1991), host-encoded mechanisms involved in conjugation control of BHR plasmids, if existing, should be highly conserved. For this reason, host factors required for conjugation of BHR plasmids would be robust targets for the development of compounds useful in the AbR dissemination battle. Screening the Keio collection of single-gene deletion mutants could provide both potential targets (by looking at functions enhancing conjugation) and potential barriers (by looking at functions reducing conjugation). Essential genes were not considered as targets for conjugation control due to the consequent negative effects on bacterial growth.

The primary HTC assay did not allow the identification of any donor function essential for R388 conjugation. Specifically, no Keio mutant inhibited transfer to less than 20 % or

increased transfer more than 2.4-fold. In contrast, controls of reduced donor ability included in **Fig 1** showed relative CF of 0.03 ± 0.01 in the presence of 2-HDA and 0.74 ± 0.03 when TrwA regulator was overexpressed in donors. TrwA regulatory protein overexpression in donors containing pSU1545 decreases 10-fold R388 conjugation using conventional conjugation assays (Moncalian *et al.* 1997). Accordingly, our confirmation assays showed CF of 0.17 after normalizing with a donor carrying the empty plasmid pKK223-3 used for *trwA* cloning ($p < 0.001$). However, HTC results showed a smaller reduction (relative CF = 0.74 ± 0.03). Considering that TrwA acts at donor level, like potential mutants affecting conjugation, HTC assay could not be sensitive enough to detect donor mutants with a slight effect on conjugation, as with TrwA effect. Whereas the first R388 transfer step from donor mutants would occur at different rates depending on the deleted gene, once R388 reaches recipient cells, the new transconjugants would act as wt donors in subsequent conjugation rounds. It is likely that this happens during the long mating needed to achieve a good fluorescence signal, causing a blur effect of the primary conjugation round. Nevertheless, a mutant with serious difficulties to act as donor would get blocked in the first conjugation round, thus showing a low fluorescence signal even after six hours. In order to discard this and other undesired effects caused by the HTC assay, conventional plate-conjugation assays during 1 h mating using a 1:10 donor-recipient ratio will allow a more accurate estimation of CF.

Among the 20 donor mutants producing the most drastic effects on the primary HTC assay, 9 significantly inhibited conjugation by 10-fold using plate-conjugation assays (**Tables 1** and **2**). The mutant *mdtA*$^{-}$, defective in part of a multidrug efflux system, was the donor that inhibited conjugation the most (relative CF = 0.07). In addition, deletion of other membrane-related genes, such as those encoding the putative phospholipase RssA, the lipoprotein YcaL, or the putative inner membrane transport protein YdfJ, diminished conjugation up to 10 times. Similarly, membrane-related functions were involved in recipient ability of R388 and ICE*Bs1* (Perez-Mendoza and de la Cruz 2009, Johnson and Grossman 2014). Together, these results suggest that the composition of bacterial membrane, both in donor and recipient cells, has an effect on conjugation efficiency, possibly affecting mating pair formation. In fact, recipient LPS was identified as the specific receptor for the PilV adhesin of IncI plasmid R64 during liquid conjugation (Ishiwa and Komano 2000) and the outer membrane protein OmpA was reported to interact to F plasmid TraN for mating pair stabilization (Klimke *et al.* 2005).

Interestingly, the mutation of four different membrane-bound subunits of ATP synthase (*atpB*, *atpD*, *atpA*, and *atpE*) inhibited R388 conjugation as well (**Table 1**). Since several ATPases are required for conjugation (Chandran Darbari and Waksman 2015), a deficient ATP supply in aerobic conditions could inhibit the process. Despite the

lower growth rate of these mutants, which produce ATP through fermentation, CF per donor was lower than the control (*polA*$^-$ undisrupted strain as donor), thus indicating a direct or indirect effect on donor ability. Also involved in aerobic respiration, fumarate hydratase FumC and 2-oxoglutarate decarboxylase SucA are enzymes of the Krebs cycle. Likewise, their mutation in donor cells could be indirectly affecting ATP supply and consequently, inhibiting conjugation (**Tables 1** and **2**). Otherwise, deletion of the replication initiation repressor SeqA might result in an excess of chromosome replication, sequestering replication machinery also needed for conjugation (**Table 2**).

An *E. coli* host factor known to be involved in conjugation as part of the relaxosome architecture regulating nicking activity (Inamoto *et al.* 1994, Nelson *et al.* 1995, Howard *et al.* 1995, Moncalian *et al.* 1999) and transcription of transfer genes (Gamas *et al.* 1987) is the integration host factor (IHF). However, IHF deficient strains used as donor or recipient cells did not show a significant effect in R388 CF (Llosa *et al.* 1991). Similar results were obtained in the primary HTC screening using mutants defective in IHF alpha and beta subunits as donors. Thus, *ihfA*$^-$ mutant showed relative CF of 0.8 ± 0.1, being 0.6 ± 0.2 the results observed using *ihfB*$^-$ (**Fig 1**).

Considering the global result of this and previous works (Perez-Mendoza and de la Cruz 2009), no *E. coli* host genes in donors or recipients were essential for conjugation of the BHR plasmid R388. If any, there might be alternative genes with redundant function, or they might be essential for bacterial growth. In this sense, it should be noted that some enzymes involved in DNA metabolism of bacterial chromosome and plasmids, such as RNA polymerase, are essential for growth (Nene and Glass 1982). Another example is the essential enzyme DNA polymerase III, required for the synthesis of the complementary strand both in donors and recipients during conjugative transfer (Wilkins and Hollom 1974, Kingsman and Willetts 1978). DNA primase is encoded by some conjugative plasmids to initiate replication of the transferred strand in recipient bacteria (Merryweather *et al.* 1986). Since plasmid R388 does not present this function and *dnaG*$^-$ recipient mutant did not affect conjugation, it was suggested that primer synthesis could be carried out by host RNA polymerase (Perez-Mendoza and de la Cruz 2009). However, validation of the Keio collection detected 25 unsuccessful mutations, among which the host DNA primase gene *dnaG* was included (Yamamoto *et al.* 2009). Since this gene was not deleted from the Keio parental strain, due to its essentiality for bacterial growth (Katayama *et al.* 1989), it could be involved in R388 replication initiation in recipient cells. Similarly, the mutants *rpoD*$^-$ (RNA polymerase sigma 70 factor), *polA*$^-$ (DNA polymerase I), *parC*$^-$ (DNA topoisomerase IV), or *rho*$^-$ (transcription termination factor), were also detected as undisrupted strains (Yamamoto *et al.* 2009) and confirmed essential for bacterial growth (Nakamura *et al.* 1983, Joyce and Grindley

1984, Olivera and Bonhoeffer 1974, Das *et al.* 1976). All these essential functions, more evolutionarily conserved than non-essential genes among different bacteria (Jordan *et al.* 2002), could be involved in DNA processing of BHR conjugative plasmids. Nevertheless, their essentiality obstructs a potential application as conjugation targets and places them as targets of common antibiotics, with their collateral resistance problems.

While mutations inhibiting conjugation would reveal functions involved in conjugation useful as targets for conjugation control, mutation of genes improving conjugation could be considered natural barriers of conjugative transfer. However, none of the 20 Keio mutants selected as donors and recipients were confirmed to significantly improve R388 conjugation (**Tables 2** and **3**). These results suggested that *E. coli* lab strains like DH5α contain no non-essential genes negatively affecting conjugation, thus being proficient donors and recipients of R388 transfer.

Contrasting to *E. coli* lab strains, which acted as capable donors and recipients of R388 conjugation, clinically isolated enterobacteria could be more resistant to R388 entrance. In fact, most of the natural strains analyzed as recipients contained mechanisms to block the entry of conjugative plasmids. Specifically, using the luminescence-based HTC assay and normalizing with *E. coli* DH5α as recipient, 79 % of the original isolates inhibited R388 conjugation more than 10-fold, whereas only 39 % of the transconjugants (isolated plasmids transferred to lab strains) did so (**Fig 3**). Therefore, most of the conjugation control mechanisms of pathogenic enterobacteria seem to be encoded by the recipient chromosome. As control of reduced recipient ability, R388 entry exclusion overexpressed in *E. coli* DH5α inhibited conjugation to 0.8 % after normalizing with CF of an empty DH5α as recipient (**Fig 3**). Similar results were obtained in previous work (Perez-Mendoza and de la Cruz 2009) and confirmed by plate-conjugation assays (**Fig 5**).

Some strains inhibiting R388 conjugation by 100-fold were selected for further analysis in order to identify the mechanisms involved in conjugation control. On the one hand, several plasmids of *E. coli* strains isolated from urinary tract infections (Ejrnaes *et al.* 2011) and transferred to *E. coli* DH5α (Alvarado 2016) encoded genes for bacteriocin production, typically present in $MOB_{P51}$ and $MOB_{C11}$ plasmids (**Table 4**) (Garcillan-Barcia *et al.* 2009). These bacteriocins indirectly inhibited conjugation by killing susceptible donor cells (**Fig 4**). On the other hand, *E. coli* original strain 3065, isolated from Umeå hospital (Ejrnaes *et al.* 2011), inhibited R388 entry to 1.8 % (**Fig 3**). The inhibition mechanism is probably encoded by the bacterial chromosome, since the ApR $MOB_{P12}$ plasmid detected (Alvarado *et al.* 2012, Alvarado 2016) did not affect conjugation when

transferred to a lab strain (**Fig 5**). The result obtained in the primary HTC assay was confirmed by plate-conjugation assay, thus discarding factors affecting light emission or bacterial growth. Conjugation of the four conjugative plasmids tested (R388, pOX38, R100-1, and R751), belonging to IncW, IncFI, IncFII, and IncP groups, showed reductions from 4-log to more than 6-log (**Fig 6**), indicating that the mechanism of inhibition was broad range. Since most prokaryotic chromosomes encode RM systems as defense against invading genomes (Oliveira *et al.* 2014), a transformation test was performed to find out the contribution of RM systems in the observed effect. As a result, transformation of *E. coli* 3065 with ColE1::*KmR* and RSF1010::*KmR* was inhibited, showing a higher inhibition effect in the case of the largest plasmid (RSF1010::*KmR*). However, transformation frequency of 3065 and DH5α with the smallest plasmid (pSU19) was similar in both strains (**Fig 7**). These results suggested that the small plasmid pSU19 does not contain any restriction site targeted by the potential RM system encoded by 3065. In addition, the largest plasmid RSF1010::*KmR* could contain more restriction targets than ColE1::*KmR*, favoring digestion. When the largest plasmids were extracted from 3065 (instead of DH5α) and transformed again into 3065, they were efficiently transformed, probably due to restriction sites protection by the methylases encoded by the potential RM system. Some conjugative plasmids encode anti-restriction proteins to evade recipient restriction systems, such as ArdC of plasmid R388, which protects transferred ssDNA from endonuclease activity (Belogurov *et al.* 2000). The presence of these adaptive traits in conjugative plasmids indicates that RM systems are widespread barriers for the dissemination of conjugative plasmids.

In summary, although no *E. coli* functions were found to be essential for R388 conjugation, some donor mutants of the Keio collection decreased R388 conjugation efficiency. Among them, some membrane-related functions and those associated with ATP synthesis seemed to be involved in conjugation. No Keio mutants as donors or recipients significantly increased conjugation, suggesting that *E. coli* DH5α did not contain any barrier for conjugation. However, most clinically isolated enterobacteria blocked plasmid entry using mechanisms such as bacteriocin production or restriction systems, which inhibited conjugation by killing donor cells or digesting foreign DNA. The study of mechanisms affecting conjugation could provide key information to manipulate conjugative transfer, with the aim of combating AbR dissemination or developing transfer devices for synthetic biology.

## 1.5. Materials and methods

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this work are summarized in **Tables 5** and **6**.

**Table 5. Bacterial strains.**

| Strains | Description | Reference |
|---|---|---|
| Keio collection | *E. coli* single-gene deletion mutants | (Baba *et al.* 2006) |
| Göteborg collection | 10 isolates from University of Göteborg | (Dahlberg *et al.* 1997) |
| Ramón y Cajal collection | 81 isolates from Ramón y Cajal Hospital | (Coque *et al.* 2008, Novais *et al.* 2008, Novais *et al.* 2010, Oliver *et al.* 2005, Novais *et al.* 2006, Novais *et al.* 2007, Valverde *et al.* 2009) |
| Santa Creu i Sant Pau collection | 94 isolates from Santa Creu i Sant Pau hospital | (Mata *et al.* 2012) |
| Umeå collection | 79 isolates from Umeå hospital | (Ejrnaes *et al.* 2011) |
| Valdecilla collection | 36 isolates from Marqués de Valdecilla hospital | (Garcillan-Barcia *et al.* 2015) |
| Autonomous collection | 16 isolates from Autonomous University of Barcelona | (Alvarado *et al.* 2012, Alvarado 2016) |
| BL21 (DE3) | *E. coli* expressing T7 polymerase | (Studier and Moffatt 1986) |
| CSH53 | *E. coli* lab strain | (Miller *et al.* 1970) |
| DH5α | *E. coli* lab strain | (Grant *et al.* 1990) |
| BW25113 | *E. coli* Keio parental lab strain | (Baba *et al.* 2006) |
| MDS42 | *E. coli* lab strain with reduced genome | (Posfai *et al.* 2006) |

Bacterial strains used in this work.

**Table 6. Plasmids.**

| Plasmids | Description | Reference |
|---|---|---|
| pJC01 | Fluorescent R388 derivative | (Getino *et al.* 2015) |
| pSU2007::Tn*lux* | Luminescent R388 derivative | (Fernandez-Lopez *et al.* 2005) |
| pUC18::*lacI*$^{q}$ | Plasmid pUC18 expressing LacI$^{q}$ | (Fernandez-Lopez *et al.* 2005) |
| pSU1545 | Vector pKK223-3 expressing TrwA | (Moncalian *et al.* 1997) |
| pKK223-3 | Expression vector | (Brosius and Holy 1984) |
| pSU5024 | Vector pET3a expressing R388 Eex | (Fernandez-Lopez *et al.* 2005) |
| pET3a | Expression vector | Novagen |
| R388 | IncW conjugative plasmid | (Datta and Hedges 1972) |
| pOX38 | IncFI conjugative plasmid | (Chandler and Galas 1983) |
| R100-1 | IncFII conjugative plasmid | (Yoshioka *et al.* 1987) |
| R751 | IncP conjugative plasmid | (Thorsted *et al.* 1998) |

Plasmids used in this work.

**HTC screening.** Fluorescence-based HTC assay employed the R388 derivative plasmid pJC01 as test plasmid (Getino *et al.* 2015). In donor cells, *gfp* gene in pJC01 is not expressed. When pJC01 is transferred to recipient strain BL21 (DE3), which carries T7 RNA polymerase, GFP is produced. CF was estimated as the ratio of absolute fluorescence emitted by transconjugant cells and $OD_{600}$, as a measure of the total number of cells. CF was normalized to the mean CF of the corresponding plate or positive control. Similarly, luminescence-based HTC assay was performed as previously described (Perez-Mendoza and de la Cruz 2009). Briefly, *lux* operon under the control of *lac* promoter encoded by the R388 derivative pSU2007::Tn*lux* is repressed in donor cells (*E. coli* CHS53) by the $LacI^q$ repressor carried in the co-resident, non-mobilizable, multi-copy plasmid pUC18::*lacI*$^q$. Upon conjugation, pSU2007::Tn*lux* but not pUC18::*lacI*$^q$ is transferred to recipient cells, where light is produced. Absolute luminescence emitted by transconjugant cells was then measured as an estimated CF and normalized to the mean CF of the corresponding plate or positive control.

**Plate-conjugation assay.** Donor (d) and recipient (r) cultures in stationary phase were washed in LB-broth (Pronadisa) and mixed in a 1:1 d:r ratio unless indicated otherwise. 200 µl of the d+r mix were centrifuged and resuspended in 15 µl LB-broth. 5 µl of this mixture were placed on top of 96-well microtiter plate wells containing 150 µl LB-agar (Pronadisa) and conjugation was allowed to proceed for 1 h at 37 ºC. Bacteria were then resuspended in 150 µl M9-broth (Sigma-Aldrich) and corresponding dilutions were plated on selective media. CF was calculated as the number of transconjugant cells per donor or recipient and normalized to the mean CF of the corresponding positive controls.

**Growth inhibition test.** LB-agar plates were inoculated with a washed overnight culture of the test strain *E. coli* DH5α. Supernatants of selected recipients and DH5α control strain grown until stationary phase were sterilized using 0.22 µm syringe filters (Millipore). Separated holes were created in the inoculated plate (using a sterilized glass Pasteur pipette), where 75 µl of each filtered supernatant were spotted. Molecule diffusion was allowed during 2 h at 4 ºC. Test strain was grown overnight to form a bacterial lawn and plate was photographed.

**Transformation test.** Plasmid DNA of pSU19 (~ 2 kb) (Bartolome *et al.* 1991), ColE1::*KmR* (~ 7 kb) (Bodsch 1977), or RSF1010::*KmR* (~ 9 kb) (Butler and Gotschlich 1991) was extracted from *E. coli* DH5α or 3065 and transformed by electroporation into DH5α or 3065 competent cells. Transformation frequency was calculated as the ratio of transformed cells per competent cell and amount of plasmid DNA added.

**Statistical analysis.** Mean comparison between two different conditions was carried out by using t test tool from GraphPad Prism® (v 5.0) biostatistics software (San Diego, CA).

# 2. Fertility inhibition between conjugative plasmids

**Getino M**, Palencia-Gándara C, de la Cruz F. Manuscript in preparation.

## 2.1. Abstract

Plasmid conjugation could be controlled by mechanisms naturally displayed by bacteria. Among them, fertility inhibition systems prevent conjugation of co-resident plasmids in donor cells. The study of interactions between conjugative systems could provide useful information to fight against antibiotic resistance dissemination. In this work, conjugation ability of IncW broad host range plasmids was analyzed in the presence of a representative set of co-resident plasmids. Two potent fertility inhibition systems against R388 conjugation were discovered in the reduced version of F plasmid pOX38 and in IncI1 plasmid R64. When R388 was substituted by a synthetic version, these effects diminished two orders of magnitude, whereas the effects caused by other fertility inhibition systems encoded by IncP1α and IncX2 plasmids were maintained. This kind of knowledge could also be valuable for constructing bacterial computing devices employing conjugative plasmids as wires.

## 2.2. Introduction

Bacteria display mechanisms to control conjugative transfer. These mechanisms may be useful to prevent the dissemination of antibiotic resistance (AbR) determinants, which are mainly transferred by conjugation (Waters 1999, Norman *et al.* 2009). Natural means to inhibit conjugation can be encoded by recipient bacteria as defense systems against potentially harmful invading genomes, such as restriction-modification or CRISPR-Cas systems (Wilkins 2002, Marraffini and Sontheimer 2008). Conjugative plasmids also present functions to regulate their own transfer, thus minimizing the burden associated to constitutive expression and phage vulnerability. This is the case of the FinOP system of IncF plasmids, which can act *in trans* between related conjugative plasmids (Frost and Koraimann 2010). In addition, plasmids have exclusion systems that prevent competition between identical plasmid backbones, block uneconomical excess of conjugative transfer, and protect recipient cells from lethal zygosis (membrane damage due to an excess of cell contacts during conjugation) (Garcillan-Barcia and de la Cruz 2008).

Diverse plasmids encode fertility inhibition genes capable of diminishing conjugative transfer of unrelated co-resident plasmids. The role of these mechanisms is thought to be a better competing ability for colonization of new hosts (Gasson and Willetts 1975,

1977). For instance, the mobilizable plasmid CloDF13 (which encodes its own coupling protein) presents a fertility inhibition factor FinC that could be inhibiting the function of the helper coupling protein in favor of CloDF13 mobilization (Willetts 1980).

Five genes (*finQ*, *finW*, *finC*, *finU*, and *finV*) encoded by different plasmids (IncI1, IncF1, ColE-like, IncI1, and IncX, respectively) are known to inhibit the transfer of IncF plasmids (Gasson and Willetts 1975), *fiwA* and *fiwB* genes of IncP1α plasmids block conjugation of IncW plasmids (Fong and Stanisich 1989), *fipA* and *pifC* inhibit fertility of IncP plasmids (Winans and Walker 1985, Miller *et al.* 1985), and *osa* inhibits the transfer of *Agrobacterium tumefaciens* pTi plasmid (Close and Kado 1991). FipA and PifC, encoded by IncN and IncFI plasmids respectively, inhibit IncP coupling protein TraG (Santini and Stanisich 1998). The most studied case is Osa, encoded by IncW plasmids. Recently, Osa structure was solved and ATPase/DNase functions revealed within its active site (Maindola *et al.* 2014). These activities were key for the T-DNA degradation observed, likely involved in Osa fertility inhibition. In addition, Osa homologs ICE1056Fin and FiwA, and even the unrelated fertility factors FipA and PifC, were found to inhibit *A. tumefaciens* T-DNA transfer *in vivo*, suggesting a conserved mode of action between fertility inhibition proteins.

To inhibit AbR propagation, manipulate conjugation for biotechnological uses, or just understand bacterial conjugation, the study of interactions between co-resident plasmids could unveil fertility inhibition systems useful for any of these purposes. This work focused on broad host range (BHR) conjugative systems and their interaction network with unrelated co-resident plasmids.

## 2.3. Results

### IncW conjugation is repressed by IncFI, IncI1, IncP1α, and IncX2 plasmids

The work systematically analyzed the transfer of wild type (wt) and synthetic IncW plasmids in the presence of a set of prototype conjugative plasmids in donor bacteria. Transfer frequencies for each IncW conjugative system and co-resident plasmid were normalized to the mean value of the tested system in the absence of any co-resident plasmid. The results obtained are summarized in **Table 1**.

**Table 1. Transfer of IncW conjugative systems in the presence of different plasmids.**

| | | | Relative IncW CF or MF | | Relative co-resident CF | |
|---|---|---|---|---|---|---|
| **Co-resident** | **Inc** | **Reference** | **Wt** | **Synthetic** | **Wt** | **Synthetic** |
| **pKM101** | IncN | (Langer *et al.* 1981) | 3.4 ** | 1.2 | 0.7 | 0.4 * |
| **pOX38::*CmR*** | IncFI | (Chandler and Galas 1983) | $7 \cdot 10^{-5}$ *** | $2 \cdot 10^{-2}$ *** | 0.8 | 0.3 * |
| **R1drd-19** | IncFII | (Meynell and Datta 1967) | 1.0 | 0.2 * | 1.4 | 0.3 *** |
| **R100-1** | IncFII | (Yoshioka *et al.* 1987) | 0.8 | 0.4 | 0.9 | 0.2 ** |
| **R64** | IncI1 | (Komano *et al.* 1990) | $8 \cdot 10^{-4}$ *** | $1 \cdot 10^{-2}$ *** | 3.1 | 0.2 ** |
| **pRL443** | IncP1α | (Elhai *et al.* 1997) | $4 \cdot 10^{-4}$ *** | $6 \cdot 10^{-5}$ *** | $8 \cdot 10^{-2}$ *** | 0.4 *** |
| **R751** | IncP1β | (Thorsted *et al.* 1998) | 2.4 | 0.5 | $8 \cdot 10^{-3}$ *** | 7.8 *** |
| **pOLA52** | IncX1 | (Sorensen *et al.* 2003) | 1.0 | 0.7 | 1.3 | 4.8 * |
| **R6K** | IncX2 | (Kolter and Helinski 1978) | $7 \cdot 10^{-4}$ *** | $5 \cdot 10^{-4}$ *** | 0.4 | 0.2 ** |
| **pCTX-M3** | IncL/M | (Golebiewski *et al.* 2007) | 0.4 | 0.2 *** | 0.9 | $8 \cdot 10^{-2}$ *** |
| **drR27** | IncH | (Whelan *et al.* 1994) | 0.8 | 1.0 | 0.9 | $6 \cdot 10^{-2}$ ** |

CF or MF of wt and synthetic IncW systems in the presence of different conjugative plasmids in donor bacteria. The results show the mean of at least four independent experiments normalized to the mean value of the corresponding IncW plasmid in the absence of co-resident plasmids (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Depending on co-resident AbR, the IncW plasmids used for conjugation or mobilization (by the synthetic R388 MPF as helper) were: R388 (yellow), pIE321 (orange), pSEVA121::*MOB-CmR* (grey), pSEVA121::*MOB-ApR* (green), or pRL662::*MOB-GmR* (blue). CF of the corresponding co-resident plasmids is represented in the last two columns, normalized to the mean CF of the corresponding co-resident plasmid in the absence of the IncW plasmid. Inc, incompatibility group (Taylor *et al.* 2004).

Plasmids R388 and pIE321 were tested as wt IncW conjugative systems. R388 was substituted by pIE321, with a backbone genome 97 % identical to R388 (Revilla *et al.* 2008), when its co-resident plasmid encoded a trimethoprim resistance gene (R751 and pCTX-M3). As shown in **Table 1**, R388 conjugation was considerably affected by the presence of four different incompatibility groups. Specifically, R388 transfer was inhibited 4-log by IncFI plasmid pOX38::*CmR* (F derivative), IncI1 plasmid R64, IncP1α plasmid pRL443 (RP4 derivative), and IncX2 plasmid R6K. Except for IncN plasmid pKM101, which caused a 3-fold improvement in R388 conjugation, all other co-resident plasmids (IncFII, IncP1β, IncX1, IncL/M, and IncH) produced no significant reduction in

R388 or pIE321 conjugation. Fertility inhibition of R388 by IncP1α plasmids was caused by the known *fiwA* and *fiwB* genes (Fong and Stanisich 1989). R6K inhibited R388 transfer by an unidentified mechanism (Olsen and Shipley 1975). On the other hand, fertility inhibition caused by IncFI and IncI1 plasmids had not been reported before. Besides, and regarding the effect of R388 or pIE321 on other plasmids transfer, only IncP1 plasmids pRL443 and R751 were affected. A 2-log reduction was observed on R751 conjugation and 1-log on pRL443 transfer, similar to the R388 previously observed effect on transfer of IncP1 plasmid RP1 (Olsen and Shipley 1975).

**R388 synthetic version is 100-fold less susceptible to pOX38 and R64 fertility inhibition**

The R388 mating pair formation (MPF) apparatus was inserted into *Escherichia coli* MDS42 streamlined chromosome (Garcillán-Barcia, unpublished). R388 *MOB* genes (including R388 *oriT*) responsible of DNA processing were mobilized using the inserted MPF apparatus as helper. Transfer mediated by this synthetic IncW system was analyzed in the presence of the same set of conjugative plasmids in the donor bacteria used for IncW wt plasmids. Depending on AbR of co-resident plasmids, three IncW mobilizable plasmids were used: pSEVA121::*MOB-CmR*, pSEVA121::*MOB-ApR*, or pRL662::*MOB-GmR*. The interactions observed between synthetic IncW system and the corresponding co-resident plasmids are shown in **Table 1**. The inhibitory effects observed on wt IncW conjugation were also seen on synthetic IncW mobilization. As previously observed, R6K produced a 4-log decrease in synthetic IncW mobilization, and pRL443 a 5-log reduction, an effect 10-fold more intense than wt. However, the influence of both pOX38 and R64 on synthetic IncW transfer was approximately 2-log lower than wt. Additionally, R1drd-19 (IncFII) and pCTX-M3 (IncL/M) negatively affected synthetic IncW mobilization by 10-fold. On the other hand, all co-resident plasmids were affected by the presence in donors of synthetic IncW conjugative systems. The most affected were IncL/M and IncH plasmids, their CF diminishing 2-log. Conjugation of IncN, IncF, IncI1, IncP1α, and IncX2 plasmids was reduced by 10 times, and conjugation of IncP1β and IncX1 increased 8 and 5-fold respectively.

**Overexpression of the IncW coupling protein partially relieves R388 fertility inhibition by pOX38**

The new fertility inhibition system observed in pOX38 reduced wt IncW conjugation by 5-log, but synthetic IncW mobilization was reduced only 2-log. This variation could be due to differences in expression of the *MOB* genes. First, *MOB* genes were expressed

from wt R388 plasmid, which provides 2-3 copies per cell (Tait *et al.* 1982, Fernandez-Lopez *et al.* 2006). Then, *MOB* genes were expressed from the low copy number plasmid pSEVA121, with a RK2-based origin of replication that provides 4-7 copies per cell (Silva-Rocha *et al.* 2013, Thomas *et al.* 1984). In addition, fertility inhibition factors FipA, PifC, and Osa were previously found to target the coupling protein of the conjugative system inhibited (Santini and Stanisich 1998, Cascales *et al.* 2005). Considering these results, we decided to overexpress the R388 coupling protein (TrwB) in donors to test whether R388 conjugation levels were restored in the presence of pOX38. However, the presence of more copies of TrwB increased R388 conjugation only 6-fold comparing to the control in the absence of TrwB (**Fig 1**).

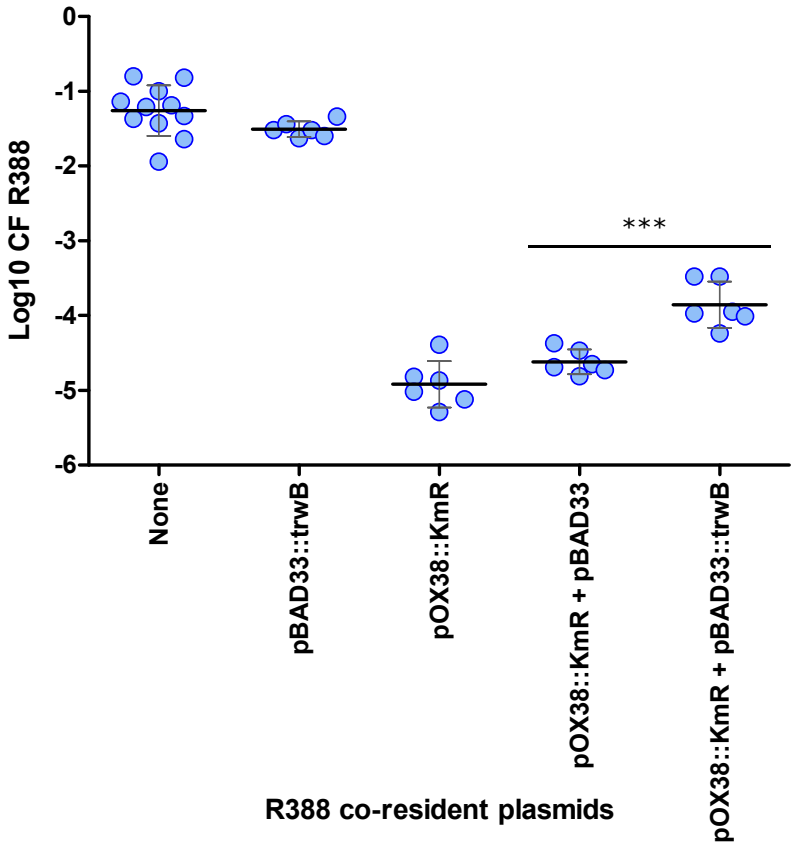



**Fig 1. R388 conjugative transfer in the presence or absence of pOX38 and R388 coupling protein TrwB.** Each point shows the CF of one experiment. Horizontal and vertical bars represent the mean ± SD obtained for each group of data (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). None, R388 CF alone. pBAD33::*trwB*, R388 CF in the presence of TrwB overexpressed. pOX38::*KmR*, R388 CF in the presence of pOX38::*KmR*. pOX38::*KmR* + pBAD33, R388 CF in the presence of pOX38::*KmR* and pBAD33 vector without *trwB*. pOX38::*KmR* + pBAD33::*trwB*, R388 CF in the presence of pOX38::*KmR* and TrwB overexpressed.

**IncX1 synthetic plasmid pX1.0 is more susceptible to fertility inhibition than the wt version pOLA52**

The synthetic version of pOLA52, pX1.0, was constructed to represent a minimal IncX1 backbone (Hansen *et al.* 2011). Conjugative transfer of wt and synthetic IncX1 plasmids, pOLA52 and pX1.0, was tested in the presence of four different co-resident plasmids with compatible AbR: IncW plasmid R388, IncFI plasmid pOX38, IncI1 plasmid R64 and IncP1β plasmid R751. Results of these experiments are shown in **Table 2**.

**Table 2. Transfer of IncX1 conjugative systems in the presence of different plasmids.**

| | | | Relative IncX1 CF | | Relative co-resident CF | |
|---|---|---|---|---|---|---|
| **Co-resident plasmid** | **Inc** | **Reference** | **Wt** | **Synthetic** | **Wt** | **Synthetic** |
| **R388** | IncW | (Datta and Hedges 1972) | 1.3 | 1.1 | 1.0 | 4.1 ** |
| **pOX38::*KmR*** | IncFI | (Chandler and Galas 1983) | 0.9 | 0.5 | 0.5 | 1.6 |
| **R64** | IncI1 | (Sampei *et al.* 2010) | 1.1 | 0.1 * | 0.9 | 0.6 |
| **R751** | IncP1β | (Thorsted *et al.* 1998) | 0.3 | $6 \cdot 10^{-2}$ *** | 1.1 | 1.2 |

CF of wt and synthetic IncX1 systems in the presence of different conjugative plasmids in donors. Results show the mean of at least four independent experiments, normalized to the mean CF of the corresponding IncX1 system in the absence of co-resident plasmids (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The plasmids used for IncX1 conjugation were: pOLA52 (wt) and pX1.0 (synthetic). CF of the corresponding co-resident plasmids is represented in the last two columns, normalized to the mean CF of the corresponding co-resident plasmid in the absence of the IncX1 plasmid. Inc, incompatibility group (Taylor *et al.* 2004).

In general, small effects were observed. The presence of IncP1β plasmid R751 caused a significant reduction (100-fold) in synthetic IncX1 conjugation. In addition, IncI1 plasmid R64 decreased 10 times pX1.0 transfer. On the contrary, the effect of these plasmids on pOLA52 was not significant. Curiously, synthetic IncX1 significantly increased by 4-fold R388 conjugative transfer.

## 2.4. Discussion

Incompatibility group W comprises conjugative plasmids with BHR, capable of carrying AbR genes to distantly related bacteria (Mazodier and Davies 1991). In addition, their relatively simple genetic organization (Fernandez-Lopez *et al.* 2014) and widespread MPF system (Christie *et al.* 2014), place them as potential conjugative wires for bacterial computing (Goni-Moreno *et al.* 2013, Amos *et al.* 2015). Therefore, the study of plasmid interactions between IncW and co-resident plasmids seems important for their application as biotechnological tools or to find new barriers against AbR propagation.

In this work, we found new fertility inhibition systems affecting IncW conjugation, encoded by the F-derived plasmid pOX38 (Guyer *et al.* 1981) and IncI1 plasmid R64. The observed effects (about 4-log reduction in both cases) fell to 2-log when R388 was substituted by its synthetic version (**Table 1**). Plasmid pSEVA121 carrying R388 *MOB* genes might produce more coupling protein than wt R388, which has been proposed as the target of other fertility inhibition systems (Santini and Stanisich 1998, Cascales *et al.* 2005). However, TrwB overexpression did not cause a complete restoration of R388 conjugation in the presence of pOX38, but only a 6-fold increase (**Fig 1**). Therefore, stoichiometric increase of other *MOB* genes, such as *trwA*, *trwC*, or even *oriT*, could be important for improving synthetic R388 transfer. In addition, *MPF* genes are inserted into the *E. coli* chromosome, which provides just one copy. Since R388 copy number is higher than one, its synthetic version would have an even higher excess of *MOB* genes comparing to *MPF* genes. This means that the number of MPF structures to transport DNA would be limited compared to the number of MOB proteins able to process DNA for conjugation. If the target was one of these MOB proteins, their excess in the synthetic R388 might make it more resistant to pOX38 inhibition than wt. As suggested for Osa mechanism of inhibition, the target could be the mobilized DNA itself (Maindola *et al.* 2014). If it was the case, an increase in DNA processing proteins and DNA itself might protect plasmid DNA from nucleases like Osa, favoring mobilization. In this sense, the isolation and sequencing of R388 mutants escaping fertility inhibition could reveal the specific targets involved.

Curiously, R64 encodes a RepC protein 99 % identical to pOX38 PifC (also called RepC), which is responsible of IncP fertility inhibition (Miller *et al.* 1985). In fact, R64 was also able to inhibit RP4 conjugation when present in the same donor (Datta *et al.* 1971). Fertility inhibition proteins, related or not, acted similarly on pTi T-DNA (Maindola *et al.* 2014). Likewise, RepC proteins encoded by F and R64 might be inhibiting fertility of both IncP and IncW plasmids. Accordingly, plasmids R1drd11 and R100-1, without a

RepC homolog, did not inhibit R388 conjugation (**Table 1**). Moreover, the similar behavior of pOX38 and R64 on wt and synthetic R388 transfer suggests a common mechanism of inhibition, different from IncP1α and IncX2. Nevertheless, previous work showed that the presence of pKM101 FipA or F PifC does not affect R388 conjugation (Santini and Stanisich 1998).

Previous data about fertility inhibition factors involving IncW plasmids were confirmed in this study (**Table 1**). Specifically, IncP1α plasmid pRL443 (with *fiwA* and *fiwB*, unlike IncP1β plasmid R751) and IncX2 plasmid R6K diminished 4-log the transfer of wt and synthetic IncW system (Olsen and Shipley 1975). Additionally, conjugation of IncP plasmids pRL443 and R751 was reduced 1 to 2-log in the presence of wt IncW plasmids (Fong and Stanisich 1989, Olsen and Shipley 1975). However, R388 synthetic version caused a smaller effect in conjugation of IncP plasmids than wt. While pRL443 transfer was reduced just to 0.4, R751 conjugation was increased 8-fold. These results suggest that the IncP inhibition system encoded by R388 and pIE321 plasmids was absent in R388 reduced version.

In addition to the four major effects affecting IncW transfer, additional minor interactions were found. For example, R1drd19 and pCTX-M3 reduced 10 times synthetic IncW mobilization (**Table 1**). Besides, the presence of synthetic IncW decreased from 1 to 2-log CF of IncN, IncF, IncI1, IncP1α, IncL/M, IncX2, and IncH plasmids. On the contrary, some plasmids slightly improved conjugation of others, such as R388 by pKM101 and pX1.0 (3 and 4-fold, respectively), or R751 and pOLA52 by synthetic IncW (8 and 5-fold, respectively). Additionally, R751 and R64 reduced the transfer of IncX1 synthetic plasmid pX1.0 (**Table 2**).

F and R64 inhibited transfer of both IncW and IncP plasmids, as observed here and in the literature (Miller *et al.* 1985, Datta *et al.* 1971). Again, R6K reduced conjugation of IncW and IncP. IncN plasmids, which encoded the FipA fertility inhibition factor against IncP plasmids, inhibited IncW plasmid pSa too. And even IncP and IncW conjugation was repressed reciprocally when present in the same donor (Olsen and Shipley 1975, Coetzee *et al.* 1972). These data suggest a general mechanism of fertility inhibition that affects many plasmids competing to invade a bacterial population, likely related to plasmid DNA degradation as suggested for Osa (Maindola *et al.* 2014). This conserved mode of action could be exploited as a means to prevent AbR dissemination. In addition, conjugative systems may be useful for future development of bacterial computing devices. In this sense, synthetic wires designed to contain the minimal number of genes needed for conjugation might prevent undesired interactions between wt plasmids, such as R388 synthetic version in the presence of pOX38 and R64 plasmids.

## 2.5. Materials and methods

**Bacterial strains and plasmids.** *E. coli* DH5α (Grant *et al.* 1990) containing different combinations of conjugative plasmids (**Table 1**) and a rifampicin-resistant derivative of *E. coli* MDS52 (Posfai *et al.* 2006) were used as donor and recipient strains respectively. Wt IncW conjugative test plasmids were R388 (Datta and Hedges 1972) and pIE321 (Gotz *et al.* 1996). Synthetic IncW mobilizable test plasmids using R388 MPF as helper inserted into *E. coli* MDS42 chromosome (Garcillán-Barcia, unpublished), were pRL662::*MOB-GmR* (pHP161) (Fernandez-Gonzalez *et al.* 2011, Vergunst *et al.* 2000), pSEVA121::*MOB-ApR* (Garcillán-Barcia, unpublished), and pSEVA121::*MOB-CmR*. IncX1 conjugative test plasmids were the wt plasmid pOLA52 (Sorensen *et al.* 2003) and its synthetic version pX1.0 (Hansen *et al.* 2011).

**Reagents.** When appropriate, antibiotics (Apollo) were added at the following concentrations: ampicillin (Ap; 100 μg/ml), chloramphenicol (Cm; 25 μg/ml), gentamycin (Gm; 10 μg/ml), kanamycin (Km; 40 μg/ml), nalidixic acid (Nx; 20 μg/ml), rifampicin (Rif; 50 μg/ml), streptomycin (Sm; 300 μg/ml), tetracycline (Tc; 10 μg/ml), and trimethoprim (Tp; 10 μg/ml). Arabinose (Sigma-Aldrich) 0.01 % was used as transcription inductor. Bacterial cultures were set up in LB-broth and LB-agar (Pronadisa). M9-broth (Sigma-Aldrich) was used to resuspend bacteria after mating and perform serial dilutions.

**Construction of pBAD33::*trwB*.** Coupling protein gene *trwB* from plasmid R388 (Datta and Hedges 1972) was amplified by PCR using the primers FKpnITrwB (CATCAGG TACCTTTAAGAAGGAGATATACATATGCATCCAGACGATCAAAGAAAG) and RHindIIITrwB (AA CAGCCAAGCTTTTAGATAGTCCCCTCAACAAAGGC), which introduce *Kpn*I and *Hind*III sites at both ends of the amplicon. The *trwB* insert and pBAD33 vector (Guzman *et al.* 1995) were digested with *Kpn*I and *Hind*III endonucleases (Thermo-Fisher). The final construct pBAD33::*trwB* was obtained after ligation and electroporation into *E. coli* DH5α competent cells.

**Construction of pSEVA121::*MOB-CmR*.** Starting from the construct pSEVA121::*MOB-ApR* (Garcillán-Barcia, unpublished), pSEVA121::*MOB* fragment was amplified by PCR using the primers pSEVA-F (ACCCCCTCCCCTCGG) and pSEVA-R (ACTC TTCCTTTTTCAATATTATTGAAGCATTTATC). *CmR* gene was amplified from plasmid pB (Garcillán-Barcia, unpublished) using the primers CmF (ATGCTTCAATAATATTGAAAAAGG AAGAGTATGGAGAAAAAAATCACTGGATATACCAC) and CmR (GGTTCCCCGATACAGCCGAG GGGAGGGGGTTTACGCCCCGCCCTG), which introduce homology regions of pSEVA121::*MOB*. The final construct pSEVA121::*MOB-CmR* was obtained through a

Gibson assembly reaction (Gibson *et al.* 2009) followed by electroporation into *E. coli* DH5α competent cells.

**Plate-conjugation assay.** Donor and recipient cultures in stationary phase were washed in LB-broth and mixed in a 1:1 donor-recipient ratio. Then, a 200 µl mix was centrifuged and resuspended in 15 µl LB-broth. 5 µl of this mixture were placed on top of 96-well microtiter plate wells containing 150 µl LB-agar and conjugation was allowed to proceed for 1 h at 37 ºC. Conjugation of drR27 was performed for 2 h at 25 ºC. Bacteria were then resuspended in 150 µl M9-broth and corresponding dilutions were plated on selective media. CF was estimated as the number of transconjugant cells per donor and means were calculated using decimal logarithms of data. Obtained results were normalized to the mean CF or MF of the corresponding system in the absence of co-resident plasmids.

**Statistical analysis.** Mean comparison between two different conditions was carried out by using t test tool from GraphPad Prism® (v 5.0) biostatistics software (San Diego, CA).

# 3. Synthetic fatty acids prevent plasmid-mediated horizontal gene transfer

**Getino M**, Sanabria-Ríos DJ, Fernández-López R, Campos-Gómez J, Sánchez-López JM, Fernández A, Carballeira NM, de la Cruz F. **MBio**. 2015 Sep 1;6(5):e01032-15.

## 3.1. Abstract

Bacterial conjugation constitutes a major horizontal gene transfer mechanism for the dissemination of antibiotic resistance genes among human pathogens. Antibiotic resistance spread could be halted or diminished by molecules that interfere with the conjugation process. In this work, synthetic 2-alkynoic fatty acids were identified as a novel class of conjugation inhibitors. Their chemical properties were investigated by using the prototype 2-hexadecynoic acid and its derivatives. Essential features of effective inhibitors were the carboxylic group, an optimal long aliphatic chain of 16 carbon atoms, and one unsaturation. Chemical modification of these groups led to inactive or less active derivatives. Conjugation inhibitors were found to act on the donor cell, affecting a wide number of pathogenic bacterial hosts, including *Escherichia*, *Salmonella*, *Pseudomonas* and *Acinetobacter*. Conjugation inhibitors were active in inhibiting transfer of IncF, IncW and IncH plasmids, moderately active against IncI, IncL/M and IncX plasmids and inactive against IncP and IncN plasmids. Importantly, 2-hexadecynoic acid avoided the spread of a derepressed IncF plasmid into a recipient population, demonstrating the feasibility of abolishing the dissemination of antimicrobial resistances by blocking bacterial conjugation.

## 3.2. Importance

Diseases caused by multidrug-resistant bacteria are taking an important toll on human morbidity and mortality. The most relevant antibiotic resistance genes come to human pathogens carried by plasmids, mainly using conjugation as a transmission mechanism. Here, we identified and characterized a series of compounds that were active against several plasmid groups of clinical relevance, in a wide variety of bacterial hosts. These inhibitors might be used for fighting antibiotic-resistance dissemination by inhibiting conjugation. Potential inhibitors could be used in specific settings (e.g., farms, fish factories or even clinical settings) to investigate their effect in the eradication of undesired resistances.

## 3.3. Introduction

Infections due to enterobacteria carrying antibiotic resistance (AbR) determinants are a major cause of global morbidity and mortality (Hawkey and Jones 2009). Despite their ongoing success, antibiotics are becoming a progressively limited weapon to fight bacterial infections. Over the past years, few novel antibiotics have been developed and larger numbers of pathogens resistant to current treatments have arisen (WHO 2014). Since AbR mechanisms are naturally present in antibiotic-producing organisms, they can easily spread to bacterial pathogens by horizontal gene transfer (HGT). In enterobacteria, plasmid conjugation is one of the main sources of HGT, and the emergence of multi-resistant pathogens is frequently linked to the spread of conjugative plasmids. For example, worldwide dissemination of extended-spectrum beta lactamases, particularly the CTX-M enzymes, is due to mobile genetic elements, especially conjugative plasmids from the IncF group (Pitout 2010). Because AbR genes disseminate mostly by conjugation, strategies to control conjugation could provide effective means to curve AbR dissemination (Smith and Romesberg 2007, Baquero *et al.* 2011). Among the proposed alternatives to conventional antibiotics, this work focuses on the development of chemical inhibitors of bacterial conjugation.

Previous efforts to control conjugation in enterobacteria focused on two complementary lines of action. First, on chemical and biological agents acting against key molecular components of the conjugation process. One of such key components is the relaxase, the protein responsible of nicking DNA at the origin of transfer and initiating plasmid transfer. Relaxase activity was inhibited by the use of bisphosphonates (Lujan *et al.* 2007), a strategy later revealed as misleading, since these compounds were found to act as unspecific chelating agents (Nash *et al.* 2012). Another strategy involved the expression of intrabodies directed against plasmid R388 relaxase. Intrabodies were expressed in recipient cells, successfully preventing the acquisition of the conjugative plasmid (Garcillan-Barcia *et al.* 2007). However, the applicability of intrabodies in clinical or environmental settings is limited, since it requires a transgenic recipient population expressing the intrabody. Another specific target for the control of conjugation was the conjugative pilus. Certain bacteriophages attach to conjugative pili with high specificity. By exploiting the natural affinity of bacteriophage M13 for the F pilus, this bacteriophage and its protein pIII were employed to inhibit F plasmid conjugation (Lin *et al.* 2011). This strategy would be most useful if it could be extended to other types of pili.

A second line of action for developing conjugation inhibitors (COINs) involves whole cell assays, i.e., screening for compounds that produce reduced numbers of transconjugant cells in conventional conjugation assays. This approach suffers from a

major backlash: the possibility of false positives arising from compounds that do not target the conjugative machinery, but inhibit cell growth instead. Indeed, many early compounds described as COINs were later found to be growth inhibitors (Hooper *et al.* 1989, Michel-Briand and Laporte 1985, Conter *et al.* 2002). Using a luminescence-based high-throughput conjugation (HTC) assay, in combination with a secondary assay that ruled out effects on growth rates, unsaturated fatty acids (uFAs) were discovered as the first effective COINs. uFAs were found to inhibit conjugation of IncW and IncF plasmids, while cell growth was not affected (Fernandez-Lopez *et al.* 2005). Screening a library of 12,000 natural compounds (NatChem library) yielded dehydrocrepenynic acid (DHCA) as the fatty acid with the highest COIN activity (Fernandez-Lopez *et al.* 2005). However DHCA has to be extracted from tropical plant seeds (Gussoni *et al.* 1994), complicating the characterization of its COIN activity. As a result, it was unclear whether DHCA and other uFAs were potent enough to efficiently block the spread of conjugative plasmids, and the range of bacterial hosts susceptible to inhibition.

In this work, starting from the chemical structure of DHCA, we developed simple synthetic COINs in sufficient amounts to study their efficiency and range of activity. We found that synthetic 2-hexadecynoic acid (2-HDA) acts as a true COIN on a wide range of bacterial species and conjugative plasmids. Importantly, 2-HDA was able to prevent the spread of the highly infective IncF plasmid R1drd19, thus demonstrating the feasibility of using COINs to block the spread of AbR.

## 3.4. Results

### 2-HDA, an effective synthetic COIN

DHCA was identified in previous work (Fernandez-Lopez *et al.* 2005) as the most potent COIN found. DHCA is a 2-alkynoic fatty acid (2-AFAs), a class of molecules known for their bioactive properties (Carballeira *et al.* 2012, Sanabria-Rios *et al.* 2014). To test whether other 2-AFAs shared COIN activity with DHCA, we tested a number of them that were simpler and amenable for chemical synthesis (Tasdemir *et al.* 2010, Carballeira *et al.* 2012, Sanabria-Rios *et al.* 2014). 2-HDA is a 2-AFA with a chain length of 16 carbon atoms and one triple bond at C-2 (**Fig. 1A**). In addition to its previously reported activities, 2-HDA inhibited R388 conjugation to 2 % at 0.3 mM ($IC_{98}$ = 0.3 mM) (**Fig. 1B**). When five monounsaturated 2-AFAs of different chain lengths were compared to test for the influence of hydrocarbon chain length in COIN activity, 2-HDA showed an optimal chain length (**Fig. 1C**). As shown in **Figure 1C**, COIN potency follows the trend 2-HDA (16 C) > 2-octadecynoic acid (2-ODA, 18 C) > 2-tetradecynoic acid (2-TDA, 14 C) > 2-icosynoic acid (2-ICA, 20 C) > 2-dodecynoic acid (2-DDA, 12 C).

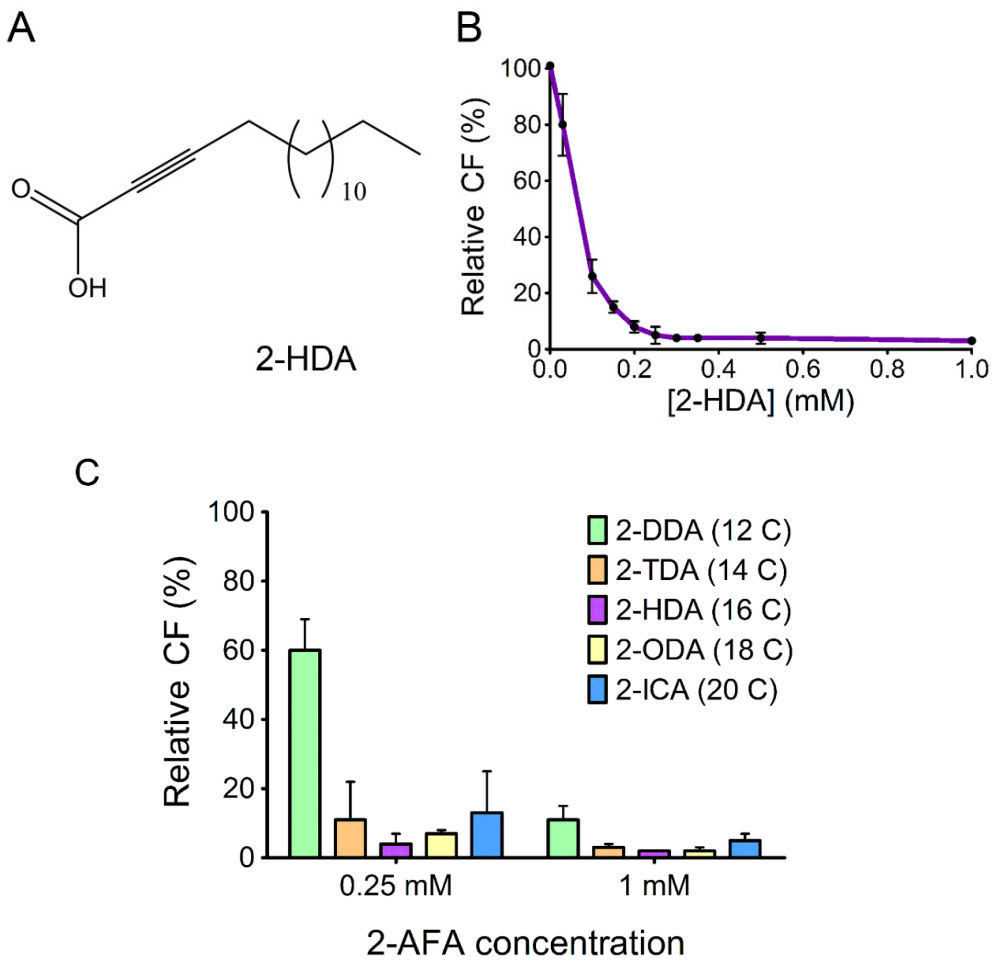



**Fig. 1. 2-Hexadecynoic (2-HDA) COIN activity.** (A) Chemical structure of 2-HDA. (B) Conjugation frequency (CF) in the presence of increasing concentrations of 2-HDA, relative to CF without 2-HDA (100 %). Values represent the mean ± SD of at least five independent experiments measured by HTC assay. (C) CF in the presence of five 2-AFAs with different chain length (12 to 20 carbon atoms) at two different concentrations, relative to CF in the absence of added compounds (100 %). The values represent the mean + SD of at least three independent experiments measured by HTC assay.

### The carboxylic group is essential to COIN activity

A set of chemical analogs of 2-HDA were synthesized to ascertain which chemical groups were crucial for the observed activity. The carboxylic group was substituted for other functional groups in the studied analogs, including 2-alkynols, methyl 2-alkynoates and tetrahydropyranyl-ethers (**Fig. 2A**). Interestingly, only 2-HDA derivatives with an unaltered carboxylic group remained active (**Fig. 2B**). The same behavior was observed with 2-ODA and its derivatives (**Fig. S1**). Moreover, two 2-HDA derivatives containing two separate triple bonds, 2,6-hexadecadiynoic acid (2,6-HDA) and 2,9-hexadecadiynoic

acid (2,9-HDA), were also assayed. Results are shown in **Figure 2**. While 1 mM 2,6-HDA inhibited conjugation at the same level as 2-HDA, when the second triple bond was placed more distantly from the carboxylic group (2,9-HDA), no inhibition was observed (**Fig. 2B**). Unlike 2-HDA, 2,6-HDA was less active at lower concentrations. For instance, 2,6-HDA inhibits conjugation only to 30 ± 4 % at 0.3 mM. In summary, a long, unsaturated hydrocarbon chain plus a carboxylic group seem to be the outstanding chemical groups required for COIN activity.

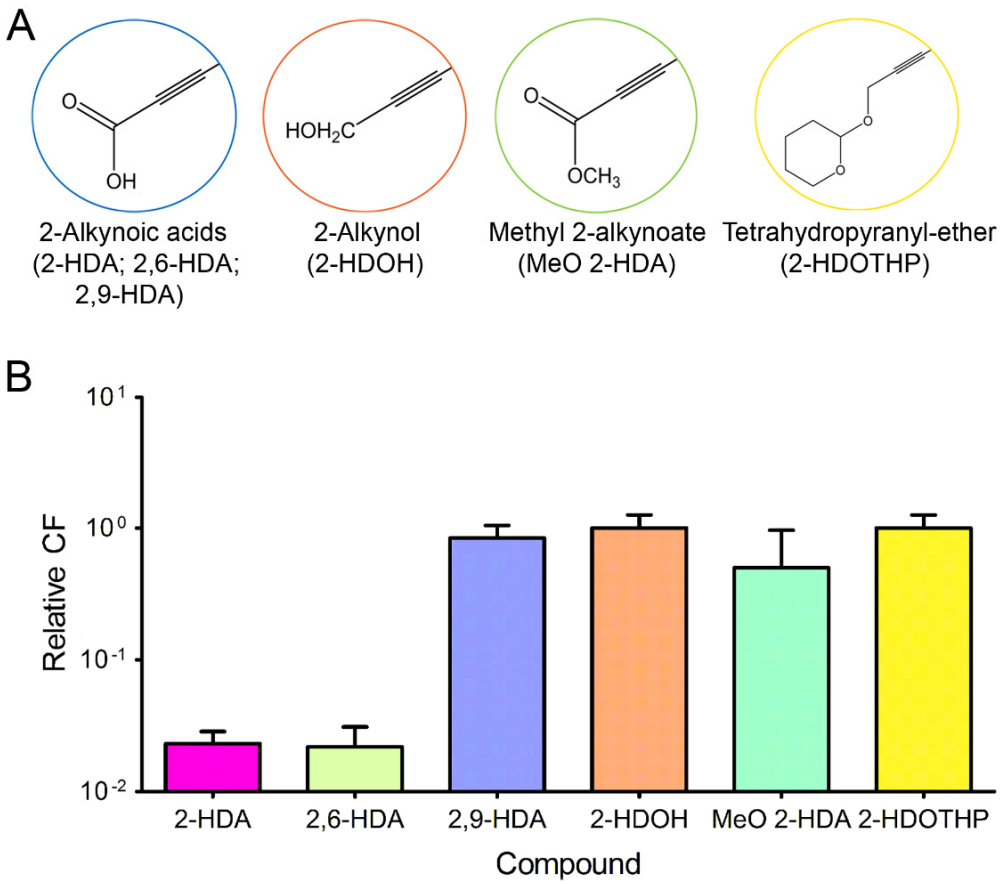



**Fig. 2. COIN activity of 2-HDA analogs.** (A) Schematic structure of functional groups of analyzed compounds. (B) Conjugation frequency (CF) represented in logarithmic scale in the presence of 1 mM 2-HDA and related compounds, relative to a control without added compounds ($10^0$). Values represent the mean + SD of at least three independent experiments measured by HTC assay.

### IncW, IncF and IncH conjugative plasmids, best targets

So far, plasmid R388 was employed to test for COIN activity, which prompts the question of how broad is the range of plasmids affected by the identified COINs. With

the purpose of expanding this scope, a collection of prototype conjugative plasmids from Enterobacteriaceae (**Table S1**) were tested with the most active 2-AFAs (2-HDA and 2-ODA). Results are shown in **Table 1**.

**Table 1. Conjugation frequency in the presence of 2-HDA and 2-ODA.**

| | | | | CF (%)[a] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 2-HDA (mM) | | | 2-ODA (mM) | |
| **Plasmid** | **Inc[b]** | **MOB[c]** | **MPF[d]** | **0.2** | **0.4** | **1** | **0.2** | **0.4** |
| **R388** | W | F11 | T | 3** | 1*** | 1*** | 29 | 9** |
| **pSa** | W | F11 | T | - | 1*** | - | - | - |
| **pIE321** | W | F11 | T | - | 1*** | - | - | - |
| **pIE522** | W | F11 | T | - | 1*** | - | - | - |
| **R7K** | W | F11 | T | - | 1*** | - | - | - |
| **pMBUI4** | W-like | F11 | T | - | 1*** | - | - | - |
| **pKM101** | N | F11 | T | 62 | 89 | 55 | 75 | 165 |
| **pOX38** | FI | F12 | F | 22* | 5*** | 3*** | 71 | 10** |
| **R1drd19** | FII | F12 | F | 11** | 3*** | 3*** | 25* | 11** |
| **R100-1** | FII | F12 | F | 5*** | 1*** | 1*** | 16* | 2*** |
| **pRL443** | P1α | P11 | T | 122 | 99 | 80 | 137 | 154 |
| **R751** | P1β | P11 | T | 117 | 55 | 154 | 57 | 55 |
| **R64drd11** | I1α | P12 | I | 90 | 14* | 4** | 51 | 47 |
| **pCTX-M3** | L/M | P131 | I | 135 | 40 | 11** | 180 | 51 |
| **R6K** | X2 | P3 | T | 47 | 27* | 17* | 57 | 26* |
| **drR27** | HI1 | H11 | F | 9*** | 6*** | 3*** | 27** | 15*** |

[a] Conjugation frequency (CF) in the presence of 2-HDA and 2-ODA using a representative set of conjugative plasmids, expressed as a percentage relative to a control without added COINs (100 %). Values represent the mean of at least four independent experiments measured by plate-conjugation assay. Mean significantly different from control with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The hyphen represents the absence of data at these concentrations. [b] Inc, incompatibility group (Taylor *et al.* 2004). [c] MOB, MOB group (Garcillan-Barcia *et al.* 2009). [d] MPF, mating pair formation type (Guglielmini *et al.* 2011).

As occurred with previously tested uFAs (Fernandez-Lopez *et al.* 2005), conjugation of IncW and IncF plasmids was preferentially inhibited in the presence of 2-AFAs. Specifically, 2-HDA reduced about 100 times conjugation frequency of IncW, IncF and IncH plasmids at a concentration of 0.4 mM (**Table 1**). In addition to R388, the conjugation of a number of $MOB_{P11}$/IncW plasmids such as pSa, pIE321, pIE522, R7K

and the IncW-like plasmid pMBUI4, were affected to the same extent as R388 itself (**Table 1**). When higher concentrations of 2-HDA were used (1 mM), IncI, IncL/M and IncX plasmids were also inhibited to various extents. Other plasmids (IncN and IncP) were not significantly affected even at the higher tested 2-HDA concentrations (**Table 1**). Hence, significant differences were observed in the sensitivity of different plasmid conjugation systems to the tested COINs, which could provide valuable insights regarding to their mode of action.

### Effect of COINs on plasmid mobilization

In addition to conjugative plasmids, mobilizable plasmids are also transmissible by conjugation, if helped by a conjugative plasmid coexisting in the donor cell (Francia *et al.* 2004). Thus, it seemed interesting to test for the transfer of different mobilizable plasmids in the presence of diverse conjugative systems. This experiment will also help to elucidate the 2-AFA target in the conjugation machinery. Thus, several mobilizable plasmids were tested in the presence of either 2-HDA or 2-ODA, using different helper plasmids. As shown in **Table 2**, only when the helper plasmid was itself affected by COINs, conjugation of the mobilizable plasmid was inhibited. In contrast, when the mobilizable plasmid ColE1 was transferred using a COIN-resistant plasmid (the IncP plasmid pRL443), its mobilization was not affected (**Table 2**).

**Table 2. Mobilization frequency in the presence of 2-HDA and 2-ODA.**

| | | | | MF (%)[a] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 2-HDA (mM) | | | 2-ODA (mM) | |
| **Plasmid[b]** | **Inc[c]** | **MOB[d]** | **MPF[e]** | **0.2** | **0.4** | **1** | **0.2** | **0.4** |
| **CloDF13 (R388)** | ColE | C11 | - (T) | 12** | 9*** | 2*** | 29 | 26* |
| **ColE1 (pOX38)** | ColE | P5 | - (F) | 8** | 4*** | 3*** | 31* | 11** |
| **ColE1 (pRL443)** | ColE | P5 | - (T) | 100 | 77 | 70 | 100 | 87 |
| **RSF1010 (pRL443)** | Q1 | Q11 | - (T) | 84 | 58 | 59 | 70 | 66 |

[a] Mobilization frequency (MF) in the presence of 2-HDA and 2-ODA using three different mobilizable plasmids, expressed as a percentage relative to a control without added COINs (100 %). Values represent the mean of at least four independent experiments measured by plate-conjugation assay. Mean significantly different from control with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. [b] In brackets, helper plasmids used. [c] Inc, incompatibility group (Taylor *et al.* 2004). [d] MOB, MOB group (Garcillan-Barcia *et al.* 2009). [e] MPF, mating pair formation type (Guglielmini *et al.* 2011). The hyphen represents the absence of MPF in mobilizable plasmids, which uses helper MPF (in brackets).

These results suggest a shared target of 2-AFAs in mobilization and conjugation, probably being part of the mating pair formation system (MPF). An additional experiment to test this hypothesis was performed by mobilizing the *oriT*-MOB region of R388 (pHP161) (Fernandez-Gonzalez *et al.* 2011) using the MPF system of plasmid pKM101 (Draper *et al.* 2005). When 0.4 mM 2-HDA was added to conjugation media, no inhibition effect was observed, as occurred for plasmid pKM101 transfer itself (**Fig. S2**).

**COINs act in a broad range of donor bacteria**

Conjugation occurs when donor cells encounter recipient cells. However, which cells are the primary targets of the inhibition reaction? To answer this question, a modified conjugation inhibition assay was carried out. Donor or recipient cells were grown in the presence of 2-HDA, and conventional conjugation assays were performed in the absence of the COIN. Under these conditions, conjugation was only inhibited when donor cells were pre-incubated with 2-HDA, as shown in **Figure 3A**. Pre-incubation of recipient cells did not show any effect. This simple experiment suggested that the tested COINs act on donor rather than on recipient cells. In this sense, with the purpose of finding out whether observed COIN activity extends to other bacterial hosts besides *Escherichia coli*, various bacteria were analyzed as donors of plasmid R388. The plasmid was introduced in *Salmonella enterica*, *Acinetobacter baumannii*, *Vibrio cholerae*, *Agrobacterium tumefaciens* and *Pseudomonas putida,* and these strains were used as donors in conventional mating experiments. R388 conjugation was inhibited in all five species in the presence of 2-HDA, as shown in **Figure 3B**. In the case of *Vibrio cholerae*, the relative lack of effect seems to be due to inhibition of donor growth by 2-HDA (**Fig. 3B**). When plasmid pSLT, an indigenous IncFII plasmid from *S. enterica*, was tested in *S. enterica* - *E. coli* matings in both directions, its conjugation frequency also showed a significant reduction when the COIN was added to the mating medium (**Fig. S3**). These results imply that COINs are generally active in inter-species conjugation.

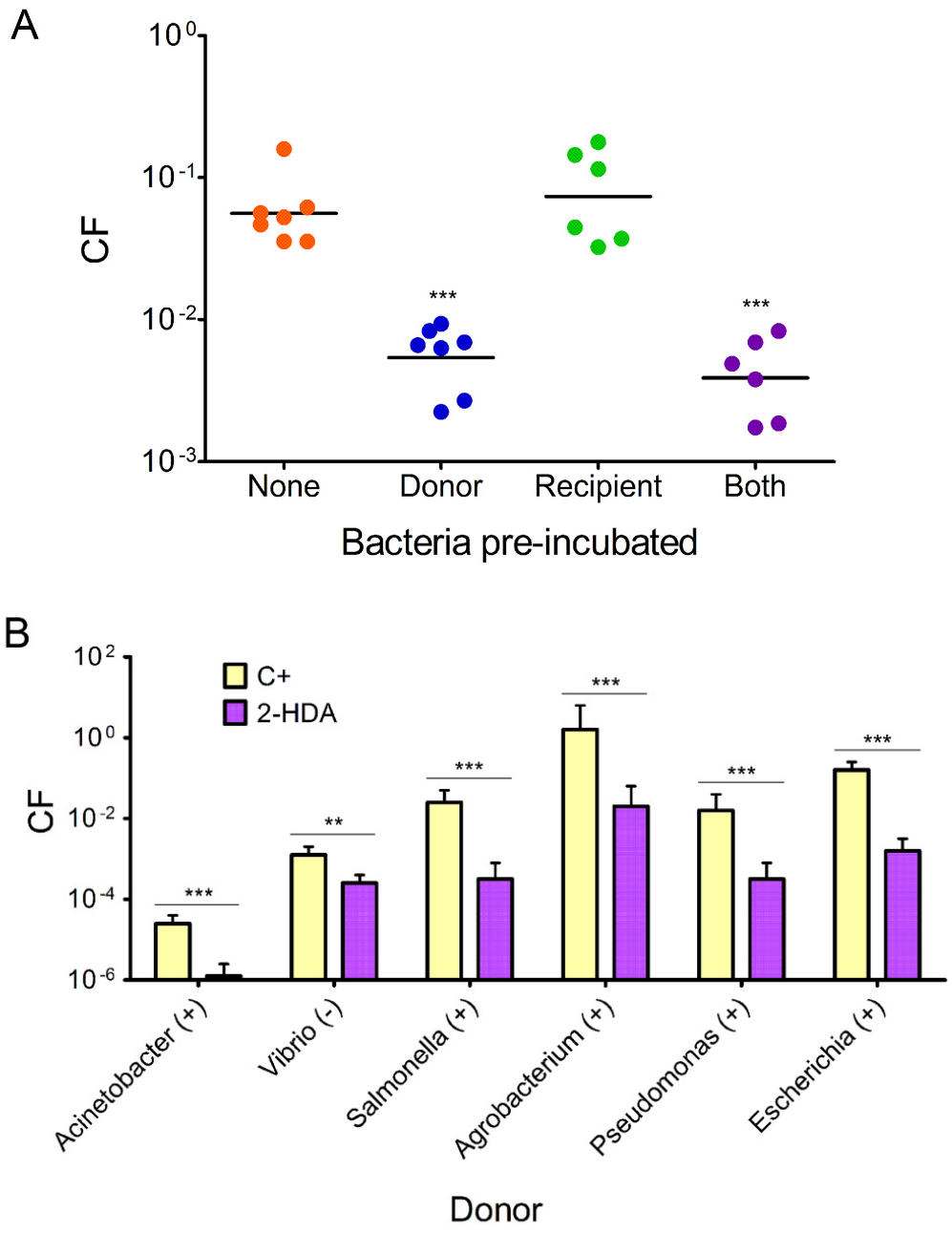
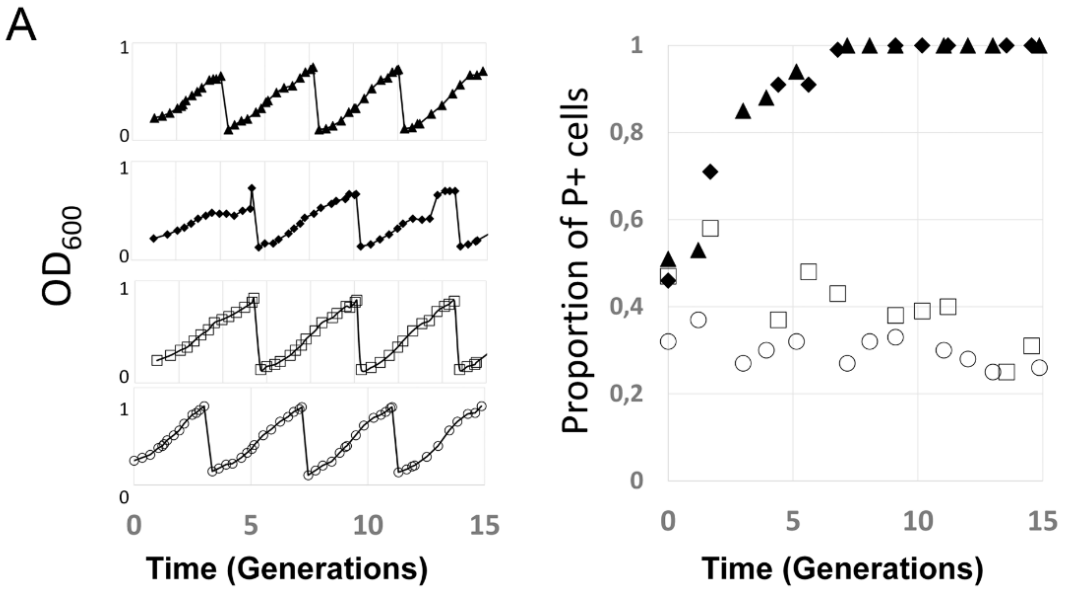



**Fig. 3. 2-HDA effect in donor bacteria.** (A) Conjugation frequency (CF) of R388 showed in logarithmic scale after either donor and/or recipient were grown overnight in the presence of 0.4 mM 2-HDA. Each point represents the result of one independent experiment measured by plate conjugation assay in the absence of COINs. Horizontal bars represent the mean value of each group of data. Mean significantly different from control (orange) with *** $p < 0.001$. (B) CF of R388 using different hosts as donor bacteria, represented in logarithmic scale in the presence of 0.4 mM 2-HDA. C+, positive control in the absence of compound. The bars represent the mean + SD of at least four independent experiments measured by plate-conjugation assay. Mean significantly different from control (yellow) with ** $p < 0.01$, *** $p < 0.001$. $OD_{600}$ of donor strains after a 24 h culture in the presence of 0.4 mM 2-HDA was similar (+) or lower (-) than control in the absence of the compound.

**2-HDA suppresses R1drd19 spread**

A key question regarding the feasibility of using COINS as an effective mean to hinder AbR dissemination is to test their effect on the spread of a conjugative plasmid in a bacterial population that contains suitable receptor cells. Plasmid spread is conditioned by the burden plasmids impose on host cells. Because this burden results in slower growth rates, plasmid-free cells tend to outcompete plasmid-bearing cells. Plasmid-bearing cells, in turn, increase their numbers by conjugation. These two processes result in a dynamic situation where the fate of a plasmid will depend on the equilibrium between plasmid infectivity and burden. This condition is classically known as the Steward-Levin Equilibrium (Stewart and Levin 1977). We investigated whether 2-HDA was able to prevent the spread of the highly infective IncF plasmid R1drd19. Plasmid R1drd19 was chosen because of its ability to conjugate in liquid at high frequencies. This allowed us to monitor plasmid prevalence in a bacterial population that started with a 1:1 donor to recipient ratio (maximal transfer rate) and was allowed to grow for 15 generations. The proportion of plasmid-containing versus plasmid-free cells was determined at different time points by replica plating, in populations that were subjected to different concentrations of 2-HDA (materials and methods). Results, shown in **Figure 4A**, demonstrated that, in the absence of 2-HDA, plasmid R1drd19 quickly overtook the population, with nearly 100 % of the cells being R1drd19$^{+}$ in 4 generations. In the presence of 400 µM 2-HDA, however, the plasmid was unable to invade the population, and its prevalence slowly decayed from 50 % to 27 % during the course of the experiment.



B

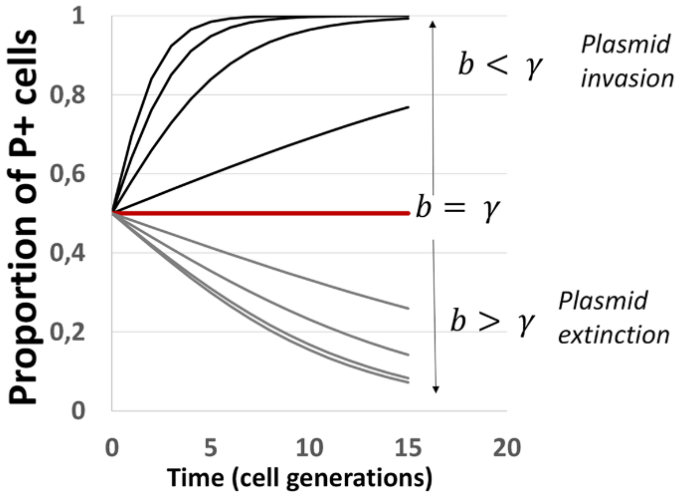



**Fig. 4. Effect of 2-HDA on plasmid R1drd19 spread in liquid medium.** (A) Donors BW27783-Nx$^r$ (R1drd19) and recipients BW27783-Rif$^r$, both in stationary phase, were mixed in 1:1 ratio and diluted either in LB (solid diamonds and solid triangles) or LB + 0.4 mM 2-HDA (empty circles and empty squares) to a final $OD_{600}$ = 0.2. Cells were incubated at 37 ºC with constant agitation (80 rpm) in turbidostatic regime, $OD_{600}$ was monitored every 10 minutes (Left panel), and when cells achieved $OD_{600}$ = 0.8, they were diluted back to $OD_{600}$ = 0.1 in LB (solid diamonds and solid triangles) or LB + 0.4 mM 2-HDA (empty circles and empty squares). In each dilution cycle, samples were taken at $OD_{600}$ 0.2, 0.4 and 0.8. Cells were diluted and plated in LB-agar without antibiotics. From these plates, 100 colonies were replica-plated on Km-containing plates to check for R1drd19 presence (Right panel). The graph shows the proportion of R1drd19 containing cells in the population (number of Km$^r$ cells / total number of cells replicated). (B) Conjugation frequency ($\gamma$) and burden on the host ($b$) determine plasmid fate. Graph shows the theoretical fraction of plasmid-containing cells along time (in cell generations) in a population of size N that at time t = 0 contained an equal number of plasmid-free ($x$) and plasmid-containing ($y$) cells. Plasmid-free cells multiply at a rate α, while plasmid-containing cells suffer from a plasmid-imposed burden b. Conjugation takes place at a frequency γ. Under these assumptions, plasmid fate depends on the magnitude of $\gamma$ - $b$. In cases where $\gamma > b$ plasmid invasion progresses and eventually overtakes the entire population. In cases where $\gamma < b$ plasmid-containing cells are driven to extinction.

To interpret these results, we built a simple ordinary differential equation model for plasmid prevalence (supplemental calculations) that includes frequency-dependent gains via conjugation and the effect of competition between plasmid-free and plasmid-containing cells in the absence of selective pressure for plasmid maintenance. Assuming a simple conjugation rate ($\gamma$) and a constant plasmid burden ($b$), the model predicts that plasmid spread will depend on the magnitude of ($\gamma$ - $b$) following:

$$\bar{Y} = \frac{e^{(\gamma-b)t}}{e^{(\gamma-b)t}+\frac{\bar{Y}_0}{\bar{X}_0}} \quad \text{Ec. 1}$$

Where $\bar{Y}$ stands for the proportion of plasmid-containing cells, and $\bar{Y}_0$ and $\bar{X}_0$ indicate respectively the proportion of plasmid-containing and plasmid-free cells at time t = 0. Exponential dependency results from the fact that transconjugants are also effective plasmid donors, thus the proportion of plasmid-containing cells progresses geometrically. Results for different ($\gamma$ - $b$) regimes are represented in **Figure 4B**, showing situations where plasmid progresses to invasion ($\gamma > b$), is driven to extinction by competition with plasmid free cells ($\gamma < b$), or remains in equilibrium ($\gamma = b$).

To characterize the effect of 2-HDA on plasmid R1drd19 conjugation frequency ($\gamma$) we measured the burden ($b$) imposed by the plasmid (**Figure S5**). We then monitored plasmid progression at different 2-HDA concentrations, and by fitting to Ec. 1 we were able to extract ($r^2 > 0.95$) the apparent $\gamma$ for each 2-HDA concentration used (**Fig. 5A**). A plot of the apparent γ values revealed an $IC_{50}$ of approximately 50 μM 2-HDA (**Fig. 5B**), equivalent to the $IC_{50}$ observed in the dose-response assays for plasmid R388 conjugation (**Fig. 1B**). Overall results indicated that 2-HDA prevented the spread of the highly infectious plasmid R1drd19 in conditions that maximize its transfer rate (exponential growth, 1:1 donor to recipient ratio). Moreover, given the burden imposed by the plasmid, in the absence of conjugation, the proportion of plasmid containing cells decayed. This indicates that 2-HDA could be used not only to block plasmid transfer into susceptible cells, but also to diminish plasmid prevalence in plasmid-containing populations.

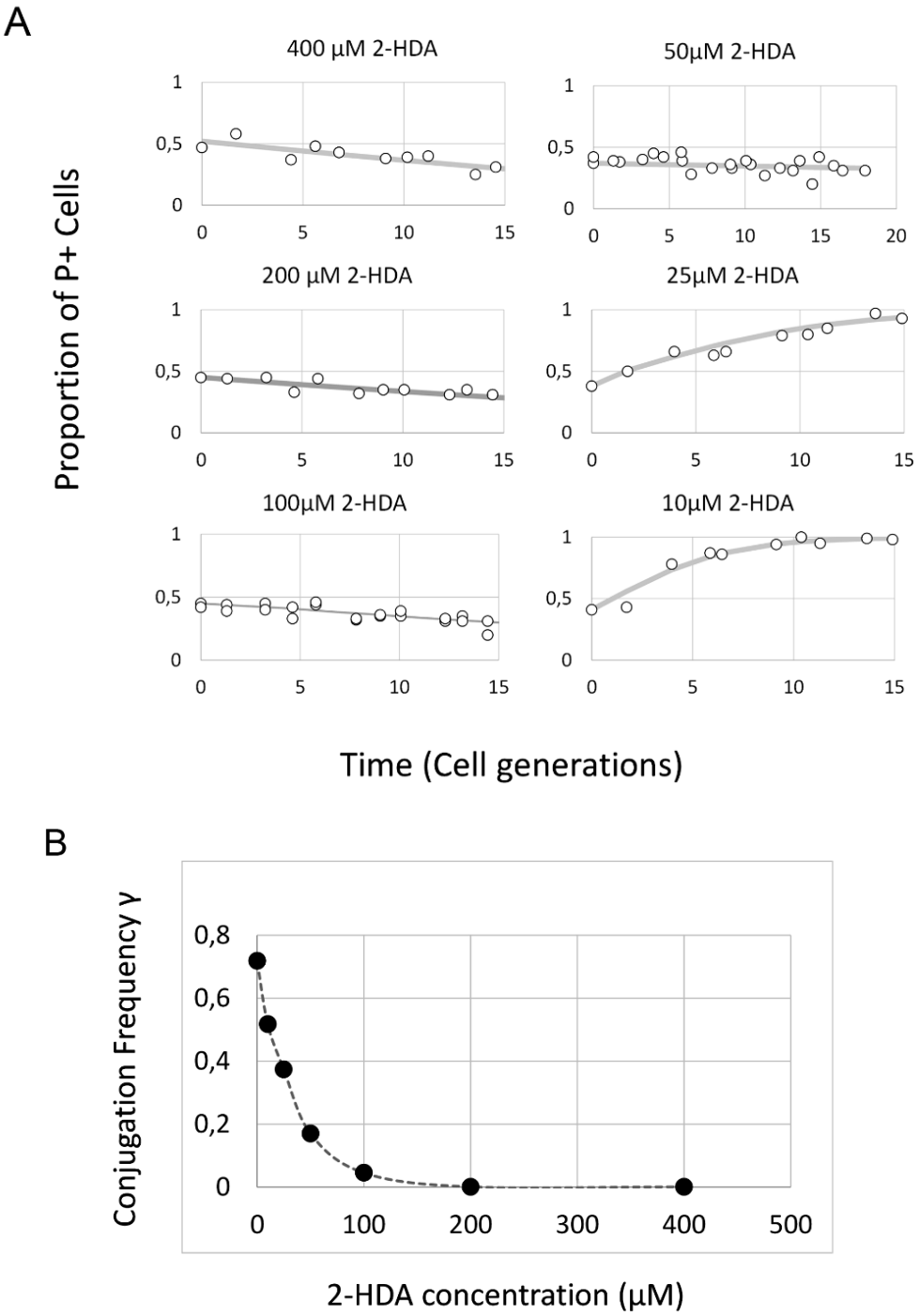



**Fig. 5. Prevention of plasmid spread is dose-dependent.** (A) Experimental result (open circles) and theoretical fit (grey lines) for plasmid spread assays using different concentrations of 2-HDA. Experimental measurements were performed as described in **Figure 4**, and the figure shows the proportion of plasmid-containing cells (x-axis) along time (y-axis, in cell generations). Non-linear least-square fitting to Ec.1 (grey lines) using the Levenberg–Marquardt algorithm was employed to determine the apparent γ values for each 2-HDA concentration used. (B) Apparent γ values (x-axis) were plotted against their corresponding 2-HDA concentrations (y-axis) to determine the dose-response curve of 2-HDA in plasmid R1drd19 spread. Results yielded an $IC_{50}$ of approximately 50 µM.

## 3.5. Discussion

The fast spread of AbRs demands effective means to stop, or at least slow down, their dissemination. Whole cell analysis demonstrated that uFAs are efficient inhibitors of bacterial conjugation (Fernandez-Lopez *et al.* 2005). However, uFAs obtained from natural sources presented a number of limitations that prevented the characterization of their COIN activity. Stable uFAs with potent COIN activity, like DHCA, were difficult to obtain from natural sources. Other available uFAs, like oleic and linoleic acid, presented lower COIN activity or were highly unstable due to auto-oxidation (Niki *et al.* 2005). Progress in COIN development required stable and easily obtainable inhibitors by chemical synthesis.

In this work we have shown that 2-AFAs, simple uFAs that can be synthesized chemically (Tasdemir *et al.* 2010, Carballeira *et al.* 2012, Sanabria-Rios *et al.* 2014), possess COIN activity. Among them, 2-HDA was the most potent COIN, with $IC_{98}$ of 0.3 mM (**Fig. 1B**), similar to that of natural uFAs (Fernandez-Lopez *et al.* 2005). As in the case of natural uFAs, 2-HDA inhibited conjugation without disrupting cell growth (**Fig. 3B**) and was effective against the same range of plasmids (**Table 1**) (Fernandez-Lopez *et al.* 2005). Altogether, data indicates that synthetic 2-AFAs are suitable substitutes for natural uFAs. Because of their synthetic nature, we were able to test the relative importance of different parts of the molecule in its COIN activity. The presence of a carboxylic group and one unsaturation proved to be essential features for COIN activity (**Fig. 2, S1**) (Fernandez-Lopez *et al.* 2005). The length of the carbon chain was also important, with 16 carbons as the optimal length for the aliphatic chain (**Fig. 1C**). The presence of other triple bonds did not increase COIN activity (2,6-HDA) or even abolished it (2,9-HDA) (**Fig. 2B**). 2-AFAs are bioactive compounds, with antifungal and even antibacterial activity against certain species (not in the case of *E. coli* or the species tested in this study except, perhaps, *V. cholerae*). Importantly, most of their bioactive properties display a similar dependency to the chemical features that correlate with potent COIN activity. Antifungal (Morbidoni *et al.* 2006), antiprotozoal (Carballeira *et al.* 2012) and antibacterial activities (Sanabria-Rios *et al.* 2014) are higher for 2-HDA and 2-ODA, the 2-AFAs that displayed the higher COIN activity (**Fig. 1C**).

In addition to potency and structural similarities, the shared spectra of action among discovered COINs (Fernandez-Lopez *et al.* 2005) (**Tables 1** and **2**) suggests a common mechanism of inhibition. A general metabolic disturbance of the bacterial cells could be invoked as the cause of inhibition. In favor of this alternative is the fact that high inhibitor concentrations are needed. At these concentrations, COINs might affect overall properties of bacterial membranes (e.g., fluidity, permeability, structural changes, etc.)

and, as a consequence, affect the function of a number of membrane proteins (including conjugation proteins). However, two sets of results are against this alternative. First, certain plasmids and not others were affected by these compounds (**Table 1**). Second, conjugation is inhibited irrespective of the bacterial host, among a variety of donors used in different experiments (**Fig. 3B, S3**). Therefore, COINs seem to target the conjugation machinery directly. Indeed, inhibition of R388 conjugation after donor pre-incubation with 2-HDA (**Fig. 3A**) suggests a specific target in the donor cell. The particular range of plasmids affected could provide valuable insights regarding their mode of action, attending to differences between them. In this respect, it is significant that uFAs affect the function of proteins associated with the bacterial membrane (Swarts *et al.* 1990, Yung and Kornberg 1988, Mahmmoud and Christensen, Haag *et al.* 1999), many of them being ATPases. Since R388 conjugation requires the active participation of at least five ATPases (TrwB, TrwC, TrwD, TrwK and StbB) (Cabezon *et al.* 2014), it is possible that uFAs specifically interact with one or several of these proteins. In fact, preliminary biochemical data from our laboratory indicate that the traffic ATPase TrwD, a component of R388 MPF system, is inhibited by linoleic acid (Machon 2004). Plasmids containing close homologs of TrwD (IncW group and related plasmids in **Fig. S4**) were also affected by these COINs. On the other hand, plasmid pKM101, which carries a TrwD homolog (TraG) incapable of replacing TrwD for R388 transfer (Ripoll-Rozada *et al.* 2013), was not affected, as shown in **Table 1**. These data are consistent with the fact that plasmids mobilized by affected conjugative plasmids, the main targets of these COINs, are also inhibited (**Table 2**), since they used MPF system of their helper plasmid for mobilization. Additionally, the absence of inhibition in a system where the *oriT*-MOB of R388 (pHP161) (Fernandez-Gonzalez *et al.* 2011) was mobilized by the MPF apparatus of pKM101 (Draper *et al.* 2005) (**Fig. S2**), also reinforces this hypothesis.

In summary, 2-AFAs provide an important scaffold structure as a starting point in the search for optimal COINs. First, their simple structures and easiness of synthesis provided sufficient amounts to analyze the key chemical features of COINs (**Fig. 2, S1**). Second, 2-AFAs shared a relatively broad range of plasmids affected (**Table 1**), among them IncF plasmids, the most common AbR carriers in pathogenic *Enterobacteriaceae*, labelled as high risk in clinical settings (Carattoli 2009). Third, analysis of 2-AFAs mode of action has revealed donors as the target cells where blockage can be installed (**Fig. 3A**) and MPF as the conjugative part affected (**Table 2, Fig. S2**), important advances into the search of the molecular target. Fourth, 2-HDA applicability study has demonstrated that conjugation can be blocked in different hosts (**Fig. 3B, S3**), and the fundamental conclusion, that observed inhibition level is sufficient to prevent AbR-carrying plasmid invasiveness in a bacterial population and even to reduce the total number of carrier cells (**Fig. 4**).

This last point is of special relevance when assessing potential applications of COINs. Although 2-HDA did not abolish conjugation at 100 %, its effect on plasmid transfer was sufficient to revert the Steward-Levin equilibrium from plasmid invasion to plasmid loss (**Fig. 4**). This indicates that even if some cells escape inhibition, the overall effect on the population is enough to prevent plasmid spread in the absence of selective pressure for plasmid maintenance. Moreover, because of the deleterious effect of plasmid burden on host fitness, COINS could be used to purge bacterial populations from transmissible plasmids. It is often observed in infectious agents that the imposed burden increases with transmissibility, with highly infective agents being usually more virulent than mildly infective ones (Levin 1996). In the case of IncF conjugative plasmids, this phenomenon is well documented (Haft *et al.* 2009). Thus, COIN action will decrease the risk of AbR spread through conjugation, while exerting a selective pressure against highly transmissible plasmids. In this regard, COINs beg to be tested in specific environments (e.g., farms, fish factories or, later, even clinical settings). The dynamics of target populations should be evaluated with COINs in the presence or absence of antibiotics to gain a firmer knowledge of their potential therapeutic utility.

## 3.6. Materials and methods

**Construction of pJC01.** Conjugative plasmid pJC01 was constructed by inserting a *gfpmut2* gene in R388 plasmid (Datta and Hedges 1972) as described in supplemental materials and methods.

**Synthesis of 2-AFAs and analogs.** The synthesis of 2-DDA, 2-TDA, 2-HDA, 2-ODA and 2-ICA followed an already published procedure (Tasdemir *et al.* 2010, Carballeira *et al.* 2012, Sanabria-Rios *et al.* 2014). 2,6-HDA and 2,9-HDA were prepared as previously described (Carballeira *et al.* 2006). 2-alkynols, methyl-ester and tetrahydropyranyl-ether derivatives of 2-HDA and 2-ODA were synthesized as shown in the literature (Sanabria-Rios *et al.* 2014, Sanabria-Ríos 2007).

**HTC assay.** A whole-cell automated assay for conjugation, based on fluorescence emission in transconjugants cells, was carried out in a Biomek3000® liquid handling robot (Beckman Coulter). Donor and recipient strains were grown until stationary phase in LB-broth with appropriate antibiotics. For surface-conjugation experiments, donor and recipient cells were concentrated 4-fold and mixed in 1:1 ratio. After that, 10 µl of each resulting conjugation mixture were spotted onto 96-well microtiter plates (Bioster), previously prepared by adding 150 µl LB 1 % agar with 1 mM IPTG and different COINs. Mating plates were incubated at 37°C for 6 h to allow conjugation, that is, the transfer of pJC01 into the recipient strain BL21 (DE3), where expression of T7 RNA polymerase

induced by IPTG triggers GFP production (**Fig. S6**). After this time, cells were resuspended in 200 µl M9 broth and 150 µl of the suspension were transferred to a new plate. The optical density ($OD_{600}$) and GFP emission of the suspensions were measured in a Victor3 Multilabel Counter (PerkinElmer). Conjugation frequencies (CF) were estimated as the ratio of absolute fluorescence emitted by transconjugant cells and $OD_{600}$ as a measurement of total number of cells. Relative CF in the presence of a compound was thus determined as a fraction of the CF in the absence of it, adding the same volume of solvent.

**Plate-conjugation assay.** For the plate-mating procedure, a 200 µl mixture of equal volumes of donor and recipient cultures previously washed, both in stationary phase, was centrifuged and resuspended in 15 µl LB-broth. Then, 5 µl of this mixture were placed on top of 96-well microtiter plate wells containing 150 µl LB-agar (± COINs) and conjugation was allowed to proceed, in general, for 1 h at 37 ºC. In the case of pSLT, mating was performed for 4 h at 37 ºC. drR27 was allowed to conjugate for 2 h at 25 ºC. *A. tumefaciens* and *P. putida* matings were carried out for 1 h at 30 ºC. Bacteria were then resuspended in 150 µl M9 broth and corresponding dilutions were plated on selective media. Conjugation frequency (CF) was calculated as the number of transconjugant cells per donor, whereas mobilization frequency (MF) was calculated as the number of cells receiving the mobilizable plasmid per donor. Since this type of frequency data were log-normally distributed, means are calculated using decimal logarithms of data. Relative CF or MF in the presence of a compound was determined as a fraction of the CF of MF in the absence of it, adding the same volume of solvent.

**R1drd19 liquid mating.** Donors BW27783-$Nx^r$ containing R1drd19 (**Table S1**) and recipients BW27783-$Rif^r$, both in stationary phase, were mixed in 1:1 ratio and diluted either in LB or LB + 0.4 mM 2-HDA to a final $OD_{600}$ = 0.2. Cells were incubated at 37 ºC / 80 rpm. Samples were taken at $OD_{600}$ 0.2, 0.4 and 0.8, when cultures were diluted to $OD_{600}$ = 0.1, allowing conjugation for three more generations (i.e., until $OD_{600}$ = 0.8 was reached again). The dilution process was repeated three times. Individual samples were appropriately diluted and plated in LB-agar without antibiotics. The resulting colonies were replica-plated on Km-containing plates to check for R1drd19 presence. Percentage of cells containing R1drd19 at each generation was calculated.

**Statistical analysis.** Mean comparison between two different conditions was carried out by using t test tool from GraphPad Prism® (v 5.0) biostatistics software (San Diego, CA).

**Phylogeny tree construction.** Phylogeny tree of $MOB_{F11}$ family relaxases was constructed by using neighbor-joining tool of MEGA5 (Tamura *et al.* 2011).

## 3.7. Supplemental figures

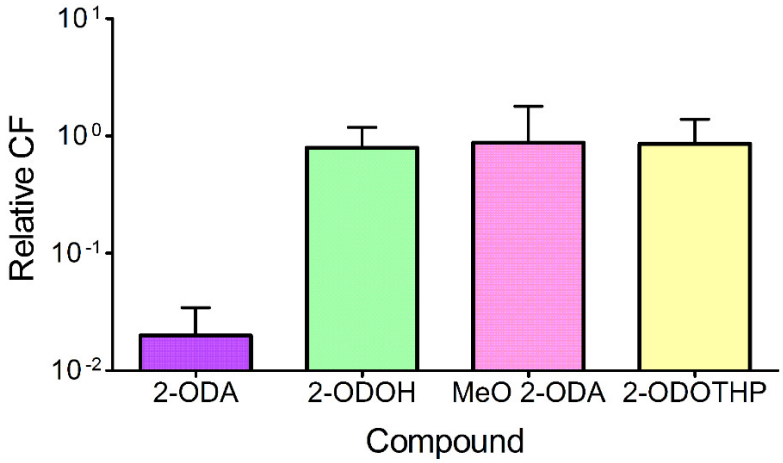



**Fig. S1. COIN activity of 2-ODA analogs.** Conjugation frequency (CF) represented in logarithmic scale in the presence of 2-ODA and derivatives at 1 mM, relative to a control without added compounds ($10^0$). Values represent the mean + SD of at least three independent experiments measured by HTC assay.

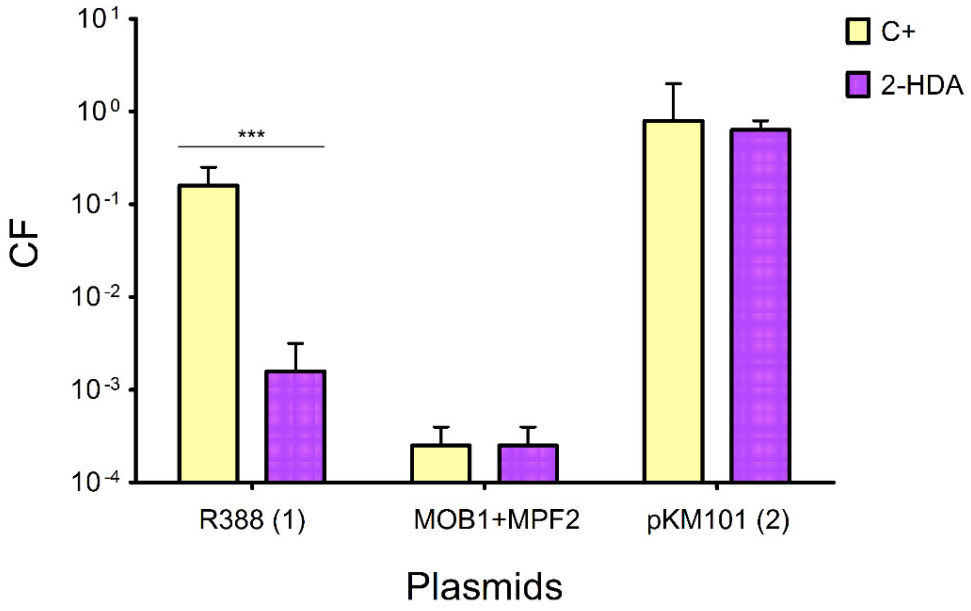



**Fig. S2. 2-HDA activity in plasmid mobilization.** Conjugation frequency (CF) of R388 and pKM101 or mobilization frequency (MF) of *oriT*-MOB of R388 (pHP161) (Fernandez-Gonzalez *et al.* 2011) using MPF apparatus of pKM101 (pKM101ΔMOB) (Draper *et al.* 2005) represented in the figure as MOB1+MPF2. Bars represent the mean + SD showed in logarithmic scale of at least three independent experiments measured by plate-conjugation assay in the presence or absence of 2-HDA 0.4 mM. C+, control in the absence of added COIN. Mean significantly different from control (yellow) with *** $p < 0.001$.

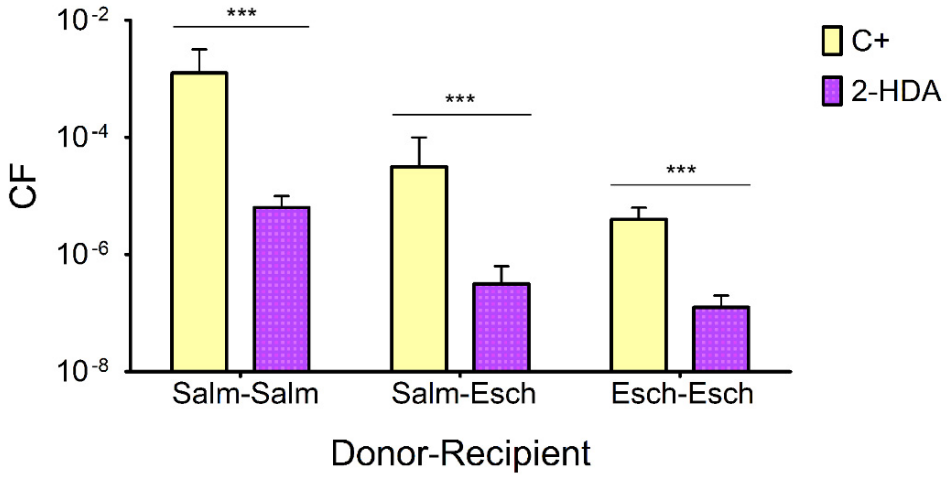



**Fig. S3. Effect of 2-HDA in conjugation of the *Salmonella enterica* indigenous plasmid pSLT.** Conjugation frequency (CF) of pSLT represented in logarithmic scale in the presence of 0.4 mM 2-HDA. Donor and recipient strains were *S. enterica* (Salm) or *Escherichia coli* (Esch). C+, control in the absence of added COIN. Bars represent the mean + SD of at least four independent experiments measured by plate-conjugation assay. Mean significantly different from control (yellow) with *** $p < 0.001$.

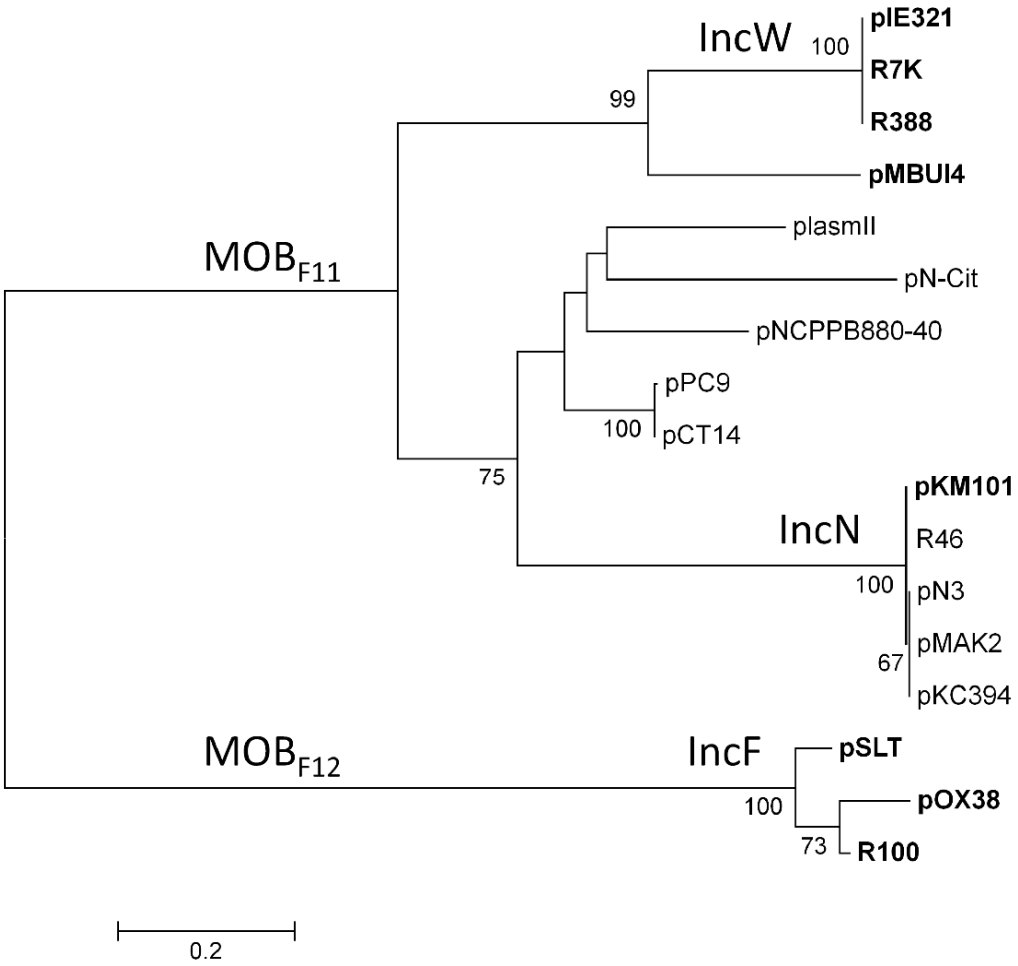



**Fig. S4. Phylogeny tree of MOB$_{F11}$ family relaxases.** MOB$_{F12}$ relaxases of plasmids pSTL, pOX38 and R100 are used as outgroups. Bootstrap percentages (over 50 %) are shown adjacent to the node being considered. Name of the relaxase-containing plasmid is shown to the right of each branch. Conjugative plasmids tested (**Table S1**) are shown in bold. MOB and Inc groups of representative plasmids are presented on corresponding branches. Accession numbers of the relaxases shown in this figure: pIE321: YP_001911166; R7K: YP_001874877; R388: CAA44853; pMBUI4: AGH89046; plasmII: CAZ15872; pN-Cit: YP_007354951; pNCPPB880-40: YP_006964196; pPC9: AGA76279; pCT14: YP_001966297; pKM101: AAB97287; R46: NP_511201; pN3: YP_004558187; pMAK2: BAF93119; pKC394: YP_003717510; pSLT: NP_490592; pOX38: BAA97974; R100: NP_052981.

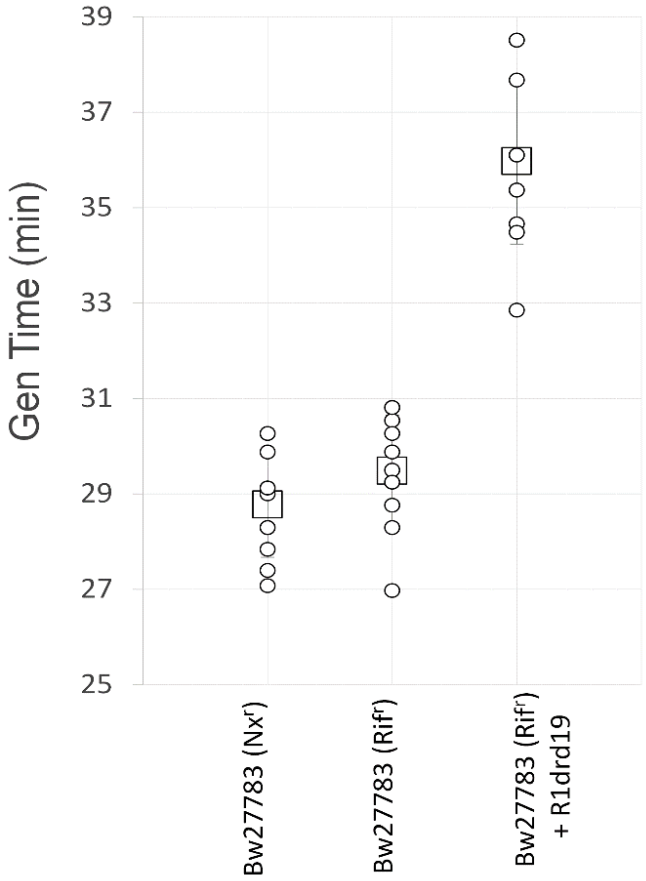



**Fig. S5**. **Plasmid burden imposed by plasmid R1drd19.** *E. coli* BW27783 ($Nx^r$ and $Rif^r$ mutants) was grown in LB medium at 37 ºC in continuous agitation in a multiwell plate reader (Victor3, Perkin Elmer). Cells were inoculated at a starting $OD_{600} \approx 0.001$ and grown for 14 h. To counteract evaporation, 6 µL of sterile distilled water was injected every 21 min in each well. $OD_{600}$ was monitored every 7 min. Resulting $OD_{600}$ were background subtracted, transformed to log scale and plotted against time. Linear fitting for the regime of exponential growth ($OD_{600}$ between 0.01 and 0.2) was employed to determine the maximal growth rate ($r^2 > 0.98$). Growth rate ($a$) was transformed into generation time ($\tau = \ln(2) / a$). The graph shows individual values (open circles) and averages and standard deviations (open boxes and error bars) of 12 growth curves. No significant differences in the generation time of nalidixic acid ($Nx^r$, first data set) and rifampicin ($Rif^r$, second data set) variants of the strain were observed. The generation time of *E. coli* BW27783 bearing de-repressed IncF plasmid R1drd19 (third data set) was found to be, on average, 7 min higher than the observed doubling time for the plasmid free strain.

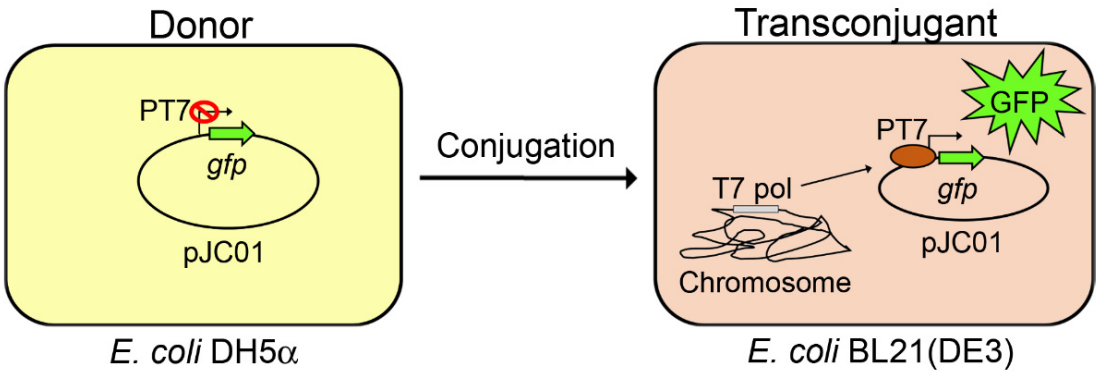



**Fig. S6**. **Schematic diagram of high-throughput conjugation assay.** T7 promoter is inactive in donor cells because it requires T7 polymerase; thus, GFP is not expressed. After conjugation, transmission of pJC01 can be detected by measuring fluorescence, since the receptor strain BL21 (DE3) express T7 polymerase allowing production of GFP. PT7, T7 promoter. T7 pol, T7 RNA polymerase. GFP, Green Fluorescence Protein.

## 3.8. Supplemental tables

**Table S1. Conjugative and mobilizable plasmids used.**

| Plasmid[a] | Inc[b] | MOB[c] | MPF[d] | Reference |
|---|---|---|---|---|
| **R388** | W | F11 | T | (Datta and Hedges 1972) |
| **pSa** | W | F11 | T | (Watanabe *et al.* 1968) |
| **pIE321** | W | F11 | T | (Gotz *et al.* 1996) |
| **pIE522** | W | F11 | T | (Gotz *et al.* 1996) |
| **R7K** | W | F11 | T | (Coetzee *et al.* 1972) |
| **pMBUI4** | W | F11 | T | (Brown *et al.* 2013) |
| **pKM101** | N | F11 | T | (Langer *et al.* 1981) |
| **pOX38** | FI | F12 | F | (Chandler and Galas 1983) |
| **R1drd19** | FII | F12 | F | (Meynell and Datta 1967) |
| **R100-1** | FII | F12 | F | (Yoshioka *et al.* 1987) |
| **pSLT** | FII | F12 | F | (Camacho and Casadesus 2002) |
| **pRL443** | P1α | P11 | T | (Elhai *et al.* 1997) |
| **R751** | P1β | P11 | T | (Thorsted *et al.* 1998) |
| **R64drd11** | I1α | P12 | I | (Komano *et al.* 1990) |
| **pCTX-M3** | L/M | P131 | I | (Golebiewski *et al.* 2007) |
| **R6K** | X2 | P3 | T | (Kolter and Helinski 1978) |
| **drR27** | HI1 | H11 | F | (Whelan *et al.* 1994) |
| **CloDF13** | ColE | C11 | - | (van Putten *et al.* 1987) |
| **ColE1** | ColE | P5 | - | (van Rensburg and Hugo 1969) |
| **RSF1010** | Q1 | Q11 | - | (Derbyshire *et al.* 1987) |

[a] Conjugative and mobilizable plasmids used in plate-conjugation assays. [b] Inc, incompatibility group (Taylor *et al.* 2004). [c] MOB, MOB group (Garcillan-Barcia *et al.* 2009). [d] MPF, mating pair formation type (Guglielmini *et al.* 2011). The hyphen represents the absence of MPF in mobilizable plasmids.

## 3.9. Supplemental material and methods

Bacterial strains and plasmids, reagents and construction of pJC01.

**Bacterial strains and plasmids.** *Escherichia coli* strain DH5α (Grant *et al.* 1990) containing conjugative plasmid pJC01 was used as donor strain and streptomycin-resistant derivative *E. coli* BL21 (DE3) (Studier and Moffatt 1986) was used as recipient strain expressing T7 RNA polymerase in HTC assay. *E. coli* DH5α (Grant *et al.* 1990)

containing different conjugative and mobilizable plasmids (**Table S1**) and a rifampicin-resistant derivative of *E. coli* MDS52 (Posfai *et al.* 2006) were used as donor and recipient strains respectively in plate-conjugation assays. *Salmonella enterica* Serovar *typhimurium* SV4522 was used as donor strain of a kanamycin-resistant derivative of pSLT plasmid (Camacho and Casadesus 2002). *Acinetobacter baumannii* ATCC 19606 (provided by Hospital de Valdecilla, Spain), *Vibrio cholera* N16961 (provided by Didier Mazel, Institut Pasteur, France), *Salmonella enterica* Serovar *typhimurium* SV4939 (provided by Dr. J. Casadesús, Universidad de Sevilla, Spain), *Agrobacterium tumefaciens* C58 (provided by Dr. D. Pérez-Mendoza, Estación Experimental del Zaidín, Spain) and *Pseudomonas putida* KT2440 (provided by Dr. F. Rojo, Centro Nacional de Biotecnología, Spain) were used as donor strains of R388. Nalidixic and rifampicin-resistant derivatives of *E. coli* BW27783 (Khlebnikov *et al.* 2002) were used as donor (containing R1drd19 plasmid) and recipient strains respectively in liquid matings. *E. coli* DH5α (Grant *et al.* 1990) containing pHP161 (Fernandez-Gonzalez *et al.* 2011) and pKM101ΔMOB (Draper *et al.* 2005) was used as donor strain to combine *oriT*-MOB of R388 and MPF apparatus of pKM101, respectively.

**Reagents.** When appropriate, antibiotics (Apollo) were added at the following concentrations: ampicillin sodium salt (Ap; 100 μg/ml), chloramphenicol (Cm; 25 μg/ml), nalidixic acid (Nx; 20 μg/ml), rifampicin (Rif; 50 μg/ml), streptomycin (Sm; 300 μg/ml), tetracycline (Tc; 10 μg/ml) and trimethoprim (Tp; 10 μg/ml). DMSO (Sigma-Aldrich) was used as solvent and IPTG (Sigma-Aldrich) as T7 RNA polymerase inductor. Bacterial cultures were set up in LB-broth and LB-agar (Pronadisa). M9 broth (Sigma-Aldrich) were used to resuspend bacteria after mating and perform serial dilutions.

**Construction of pJC01**. The *Xba*I fragment of plasmid pUA66 (Zaslaver *et al.* 2006) encoding the GFPmut2 protein (Cormack *et al.* 1996) was inserted into the *Xba*I site of plasmid pET3a (Novagen) to obtain pETGFP3, where the *gfpmut2* gene is under the control of the T7 φ10 promoter. Then, the chloramphenicol resistance gene ($Cm^r$) from pSB1C3 (http://parts.igem.org/Part:pSB1C3) was amplified by PCR using the primers $Cm^r$ sense (CGTAAGATCTTCCAACTTTCACCATAATG) and $Cm^r$ antisense (AGCTAGATCTCAAATTACGCCCCGCCCTG), which introduce *Bgl*II sites at both ends of the amplicon. The $Cm^r$ fragment was digested with *Bgl*II and inserted in the same site of pETGFP3 (flanking the *gpfmut2* gene in a divergent orientation) to obtain the construct pETGFP-Cm6. This plasmid was used as template to amplify the region containing the adjacent (and divergent) genes *gfpmut2* and $Cm^r$ by PCR using the primers Sense-EcoRI (CAGCGAATTCAGCTTCCTTTCG GGCTTTG) and Antisense-SacI (AGTGGCGAGCTCGATCTTCCCCATCGGTG). Subsequently, the amplified *gfpmut2-*$Cm^r$ cassette was digested with *EcoRI* and *Sac*I enzymes and inserted into the

conjugative plasmid R388 (Datta and Hedges 1972), previously digested with the same enzymes, to obtain the final construction pJC01.

## 3.10. Supplemental calculations

Ordinary differential equation model of Equation 1.

Equation 1 in the main text can be easily derived from the following ordinary differential equation model:

Let *N* be a population of *x* plasmid-free cells and *y* plasmid-containing cells. Plasmid-free cells grow and divide at a rate *α*, while plasmid-containing cells suffer from a burden *b*. Assuming a frequency-determined rate *γ*, and negligible plasmid losses due to mis-segregation, the equations that describe the progression of plasmid-free and plasmid-containing cells are:

$$\begin{cases} \frac{dx}{dt} = \alpha x - \frac{\gamma x y}{N} & x = \text{plasmid} - \text{free cells} \\ \frac{dy}{dt} = (\alpha - b)y + \frac{\gamma x y}{N} & y = \text{plasmid} - \text{containing cells} \end{cases}$$

We can also describe the overall growth of the population by writing:

$$\frac{dN}{dt} = \alpha x + (\alpha - b)y$$

$$\frac{dN}{dt} = \alpha x + \alpha y - by = \alpha(x + y) - by$$

$$\frac{dN}{dt} = \alpha N - by$$

We are interested in the progression of the fraction of the population that is either plasmid-free or contains a plasmid.

$$\bar{X} = \frac{x}{N}$$

$$\bar{Y} = \frac{y}{N}$$

$$\bar{X} = 1 - \bar{Y}$$

and

$$\frac{d\bar{X}}{dt} = \frac{\frac{dx}{dt}N - \frac{dN}{dt}x}{N^2} = \frac{\left(\alpha x - \frac{\gamma xy}{N}\right)N - (\alpha N - by)x}{N^2}$$

$$\frac{d\bar{X}}{dt} = \frac{\alpha Nx - \gamma xy - \alpha Nx + byx}{N^2}$$

$$\frac{d\bar{X}}{dt} = \frac{xy}{N^2}(b-\gamma) = \bar{X}\,\bar{Y}(b-\gamma) = \bar{X}(1-\bar{X})(b-\gamma)$$

and similarly

$$\frac{d\bar{X}}{dt} = \bar{X}(1-\bar{X})(\gamma - b)$$

This equation yields two trivial steady-states $\bar{X}$ = 0 and $\bar{X}$ = 1 for any parameter regime, and also the situation $b = \gamma$. Integrating over t we can determine $\bar{X}$ = f($t$)

$$\bar{Y}_t = \int_0^t \left(\bar{X}(1-\bar{X})(\gamma - b)\right) = \frac{e^{(\gamma-b)t}}{e^{(\gamma-b)t} + \frac{1-\bar{X}_0}{\bar{X}_0}}$$

For initial conditions $\bar{X}_0$ = 0.5 (equal number of donors and recipients) this expression simplifies to:

$$\bar{Y}_t = \frac{e^{(\gamma-b)t}}{e^{(\gamma-b)t} + 1}$$

# 4. Tanzawaic acids, a chemically novel set of bacterial conjugation inhibitors

**Getino M**, Fernández-López R, Palencia-Gándara C, Campos-Gómez J, Sánchez-López JM, Martínez M, Fernández A, de la Cruz F. **PLoS One**. 2016 Jan 26;11(1):e0148098.

## 4.1. Abstract

Bacterial conjugation is the main mechanism for the dissemination of multiple antibiotic resistance in human pathogens. This dissemination could be controlled by molecules that interfere with the conjugation process. A search for conjugation inhibitors among a collection of 1,632 natural compounds, identified tanzawaic acids A and B as best hits. They specially inhibited IncW and IncFII conjugative systems, including plasmids mobilized by them. Plasmids belonging to IncFI, IncI, IncL/M, IncX and IncH incompatibility groups were targeted to a lesser extent, whereas IncN and IncP plasmids were unaffected. Tanzawaic acids showed reduced toxicity in bacterial, fungal or human cells, when compared to synthetic conjugation inhibitors, opening the possibility of their deployment in complex environments, including natural settings relevant for antibiotic resistance dissemination.

## 4.2. Introduction

Infections due to antibiotic-resistant (AbR) enterobacteria are a worldwide cause of morbidity and mortality (Hawkey and Jones 2009). Moreover, the interest in developing new antibiotics by the pharmaceutical industry is declining due to high development costs and the ability of bacteria to evolve quickly and thus overcome antibiotic action (Boucher *et al.* 2009). As AbR genes disseminate mostly by conjugation (Halary *et al.* 2010, Norman *et al.* 2009), we proposed a new strategy to control AbR dissemination before infection, targeting AbR plasmid conjugation (Baquero *et al.* 2011, Fernandez-Lopez *et al.* 2005). Efforts to control conjugation include either targeting specific components (Lujan *et al.* 2007, Garcillan-Barcia *et al.* 2007, Lin *et al.* 2011) or the overall conjugation process (Getino *et al.* 2015, Fernandez-Lopez *et al.* 2005). However, only unsaturated fatty acids (uFAs) were considered effective compounds in practice to inhibit plasmid conjugation in enterobacteria (Getino *et al.* 2015, Fernandez-Lopez *et al.* 2005). Bisphosphonates, on the other hand, were recently revealed as nonspecific chelating agents (Nash *et al.* 2012) instead of specific inhibitors of plasmid F relaxase (Lujan *et al.* 2007).

Among previously discovered conjugation inhibitors (COINs), the most potent to date, dehydrocrepenynic acid (Fernandez-Lopez *et al.* 2005), is extracted from tropical plant seeds (Gussoni *et al.* 1994). uFAs, such as oleic and linoleic acids, have double bonds susceptible to oxidation (Niki *et al.* 2005). Although triple-bonded fatty acids 2-hexadecynoic acid (2-HDA) and 2-octadecynoic acid (2-ODA) are promising COINs, easily synthesized (Tasdemir *et al.* 2010, Carballeira *et al.* 2012, Sanabria-Rios *et al.* 2014) and capable of preventing plasmid invasiveness in a bacterial population (Getino *et al.* 2015), they have toxicity issues that must be overcome. Although 2-HDA showed no toxicity in *Escherichia coli*, it was found to be toxic for fungi (Gershon and Shanks 1978, Carballeira *et al.* 2006), protozoa (Tasdemir *et al.* 2010, Carballeira *et al.* 2012), gram positive bacteria, some gram-negative bacteria and eukaryotic cells (Konthikamee *et al.* 1982, Morbidoni *et al.* 2006, Sanabria-Rios *et al.* 2014). Because COINs act as prophylactic molecules, but do not elicit a direct therapeutically action, their practical application requires administration in environmental settings where plasmid conjugation occurs. Thus, COIN toxicity must be reduced, ideally completely eliminated, while chemical stability has to be maintained.

We decided to screen AQUAc, a collection of bioactive compounds isolated from aquatic microorganisms, in a search for better COINs. We expected to find compounds with different target specificity, better potency and stability, or less toxic to different cell types. The compound collection was tested by using a whole-cell automated assay. As a result, tanzawaic acids (TZAs) A and B were discovered as natural COINs with reduced toxicity compared to synthetic ones, able to inhibit bacterial conjugation of an important fraction of relevant plasmid groups.

## 4.3. Results

### High-throughput conjugation (HTC) screening of AQUAc collection

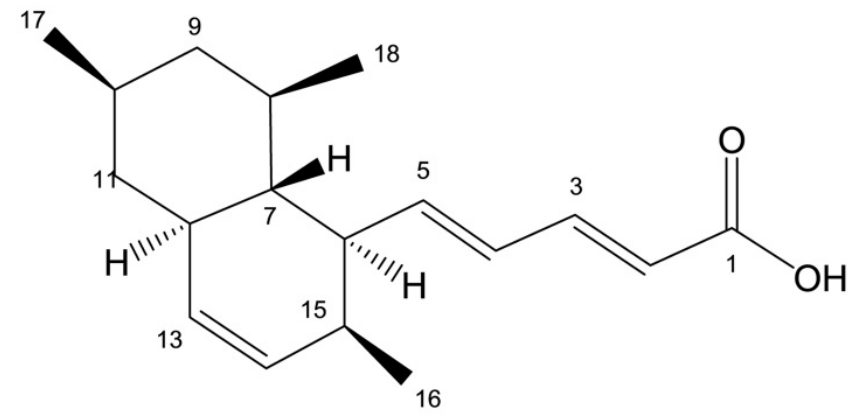
A total of 1,632 partially purified natural compounds extracted from a diversity of marine microorganisms (mainly actinomycetes, fungi and micro-algae) constitute the AQUAc collection from Biomar Microbial Technologies. It contains a high percentage of novel chemical structures (http://www.biomarmicrobialtechnologies.com). The AQUAc collection was analyzed using a luminescence-based HTC screening assay (Fernandez-Lopez *et al.* 2005). The IncW plasmid R388 was selected as the test plasmid due to its simple genetic organization (Fernandez-Lopez *et al.* 2014) and its widespread mating pair formation (MPF) system, similar to that of the well-known *Agrobacterium tumefaciens* Ti plasmid (Christie *et al.* 2014). A total of 9 compounds showed luminescence values under the selected threshold at tested concentrations and were

chosen as best hits (**S1 Fig**). Control assays were carried out to discard hits affecting bacterial growth, plasmid stability, *lux* expression or light production. None of the selected compounds (except perhaps P515) reduced luminescence of control cells containing plasmid pSU2007::Tn*lux*, which emits light constitutively (**S2 Fig**). Potency assays were subsequently carried out to select the most effective COINs (**S1 Table**). Two promising hits, P515 and P605, were selected for further analysis. Confirmation of COIN activity by plate-conjugation assays carried out in triplicate (at 50 μg/ml COIN concentration) resulted in relative frequency values of 1 % for compound P515 and 20 % for compound P605, respectively. Scale-up fermentations of the appropriate organisms were performed, bulk harvested biomass was extracted and serial HTC-guided fractionation was carried out to purify the active compounds present in P515 and P605 producer strains.

**TZAs A and B inhibit R388 conjugation**

Fractionation of extracts obtained from P515 and P605 producer strains was guided by a HTC assay based on fluorescence emission by transconjugant cells (Getino *et al.* 2015). Re-fermentation of the P515-producing strain did not allow the purification of any active compound. Guided fractionation of P605 allowed the purification of one active compound, whose structure was elucidated by nuclear magnetic resonance (**Fig 1**). The new COIN was identified as TZA-B, a polyketide previously described as inhibitor of superoxide anion production from *Penicillium citrinum* (Kuramoto *et al.* 1997, Malmstrom *et al.* 2000). Dose/response analysis of TZA-B was also performed by fluorescence-based HTC assay. As a result, 0.4 mM TZA-B was found to inhibit R388 conjugation to 2 % (**Fig 2**), as confirmed by plate-conjugation assay (2 ± 2 %).

A




B

| Position | $^{13}C$ (δ) | $^{1}H$ (δ) |
|---|---|---|
| **1** | 172.5 (C) | |
| **2** | 118.2 (CH) | 5.78 (d, 15.2) |
| **3** | 147.8 (CH) | 7.38 (dd, 15.2, 11.0) |
| **4** | 126.5 (CH) | 6.15 (dd, 15.0, 11.0) |
| **5** | 152.3 (CH) | 6.30 (dd, 15.0, 10.2) |
| **6** | 49.5 (CH) | 2.42 (ddd, 15.4, 10.2, 5.4) |
| **7** | 47.0 (CH) | 0.90 (m) |
| **8** | 36.7 (CH) | 1.35 (m) |
| **9a** | 46.9 ($CH_2$) | 0.74 (ddd, 12.7, 12.7, 12.7) |
| **9b** | 46.9 ($CH_2$) | 1.62 (ddd, 12.7, 5.4, 3.1) |
| **10** | 32.7 (CH) | 1.52 (m) |
| **11a** | 41.9 ($CH_2$) | 0.78 (ddd, 12.6, 12.6, 12.6) |
| **11b** | 41.9 ($CH_2$) | 1.70 (ddd, 12.6, 5.2, 3.1) |
| **12** | 42.9 (CH) | 1.80 (m) |
| **13** | 132.4 (CH) | 5.42 (ddd, 9.4, 1.4, 1.4) |
| **14** | 132.2CH) | 5.55(ddd, 9.4, 4.2, 2.6) |
| **15** | 37.2 (CH) | 2.20 (m) |
| **16** | 16.7 ($CH_3$) | 0.95 (d, 7.2) |
| **17** | 22.6 ($CH_3$) | 0.88 (d, 6.5) |
| **28** | 23.0 ($CH_3$) | 0.90 (d, 6.5) |

**Fig 1. Structural elucidation of TZA-B.** (A) Chemical structure of TZA-B, indicating carbon positions. (B) 1H and 13C NMR spectral data of TZA-B [δ (ppm), JHH (Hz); CDCl3].

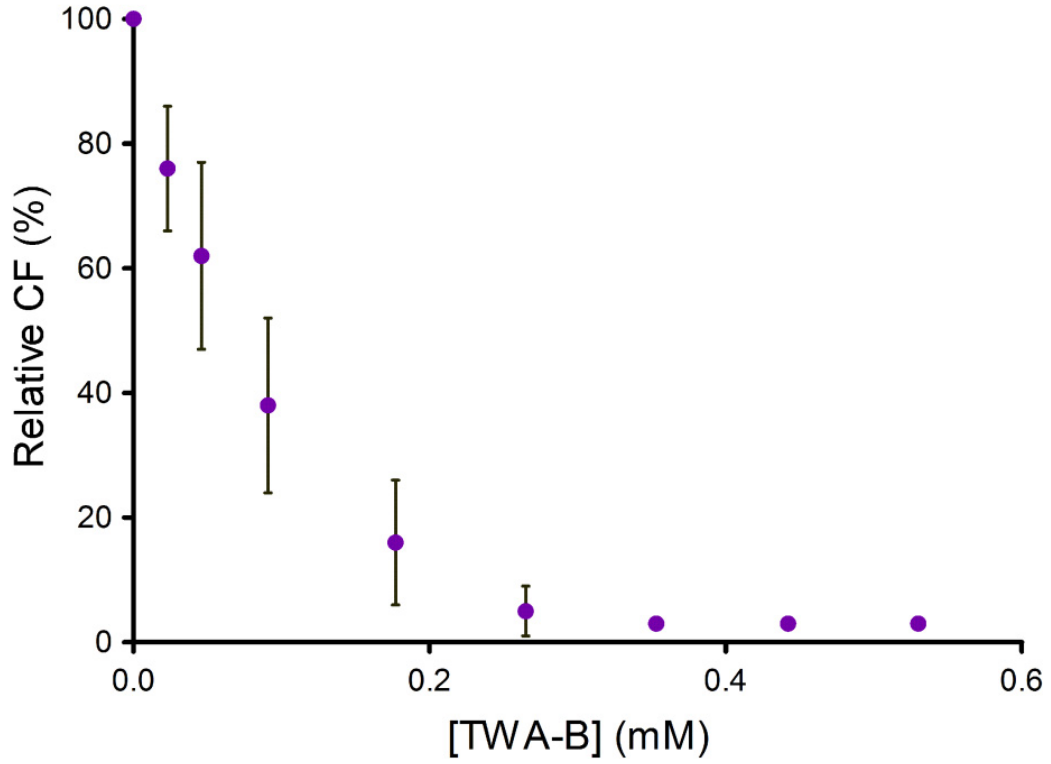



**Fig 2. Conjugation frequency (CF) in the presence of increasing concentrations of TZA-B.** Values represent the mean CF ± SD of at least four independent experiments, measured by fluorescence-based HTC assay and relative to positive control in the absence of COINs (100 %).

In the same way as TZA-B, two of its structural analogs, namely TZAs A and E (**Fig 3A**), are also inhibitors of superoxide anion production (Kuramoto *et al.* 1997, Malmstrom *et al.* 2000). They were also checked as possible COINs. While TZA-A inhibited R388 conjugation to levels similar to TZA-B, TZA-E, carrying an additional hydroxyl group in its chemical structure, did not show significant COIN activity (**Fig 3B**). Interestingly, TZA-A was present in one of the 9 hits selected in the primary HTC assay (**S1 Fig**), specifically AD0103 (**S2 Fig**), which contained 60 % pure TZA-A.

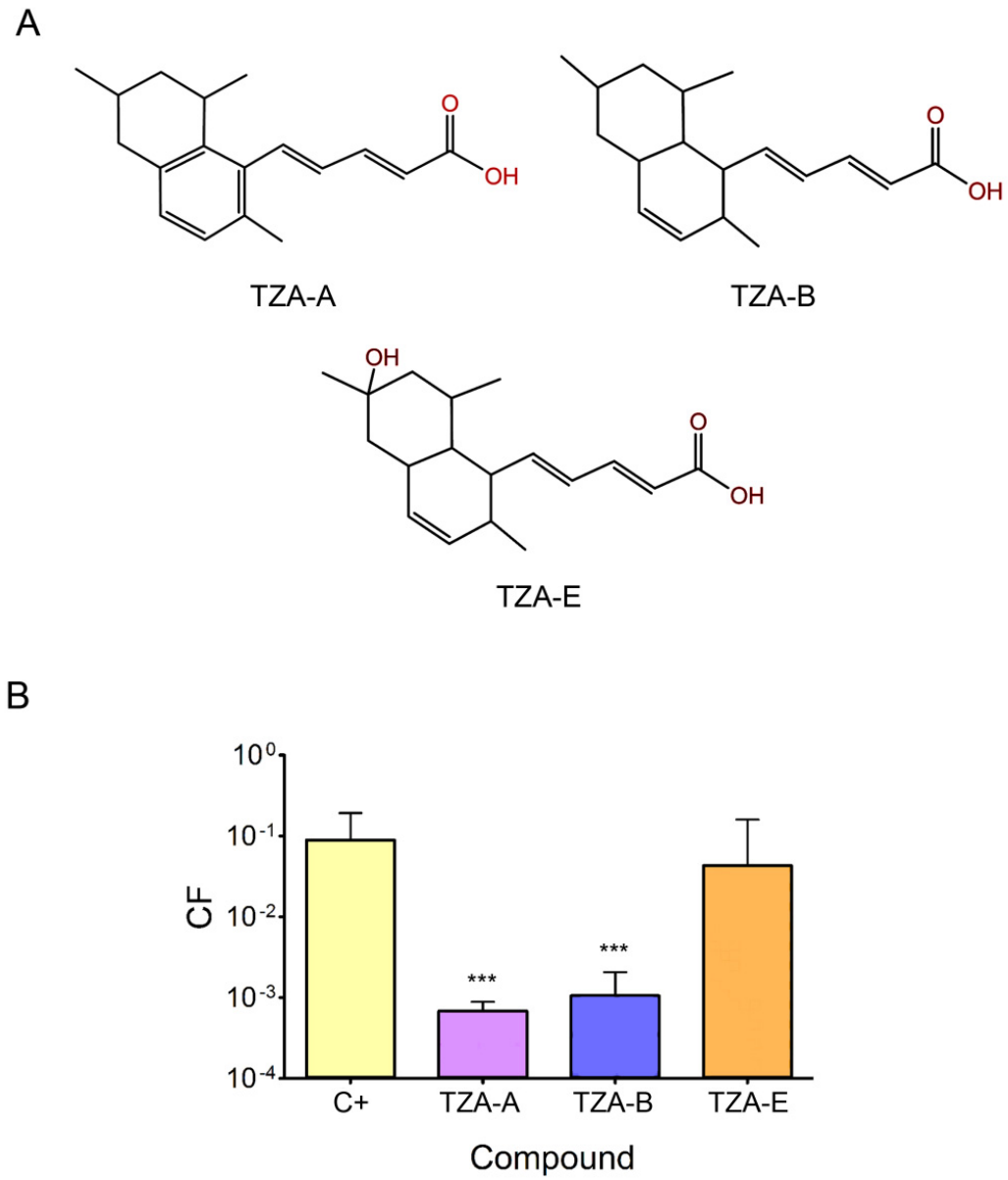



**Fig 3. TZAs A, B and E structure and activity.** (A) Chemical structure of TZAs A, B, and E. (B) CF of plasmid R388, measured by plate-conjugation assay and represented in logarithmic scale in the presence of 1 mM TZAs A, B, or E. C+, control in the absence of added compound. Bars represent the mean CF + SD of at least three independent experiments (*** $p < 0.001$).

### IncW and IncF conjugative plasmids, main targets

A collection of clinically representative conjugative plasmids found in Enterobacteriaceae was tested to investigate the range of TZA-B susceptible plasmids. Results are shown in **Fig 4**. Conjugation of the IncW plasmid R388 and the IncFII plasmid R100-1 was specially inhibited in the presence of TZA-B, almost 100-fold at 0.4 mM concentration. Besides, IncFI (pOX38), IncFII (R1drd19), IncI (R64drd11), IncL/M

(pCTX-M3), IncX (R6K) and IncH (drR27) plasmids were also inhibited, although to a lesser extent (CF from 10 to 50 %). Other plasmid groups, such as IncN and IncP, were not affected.

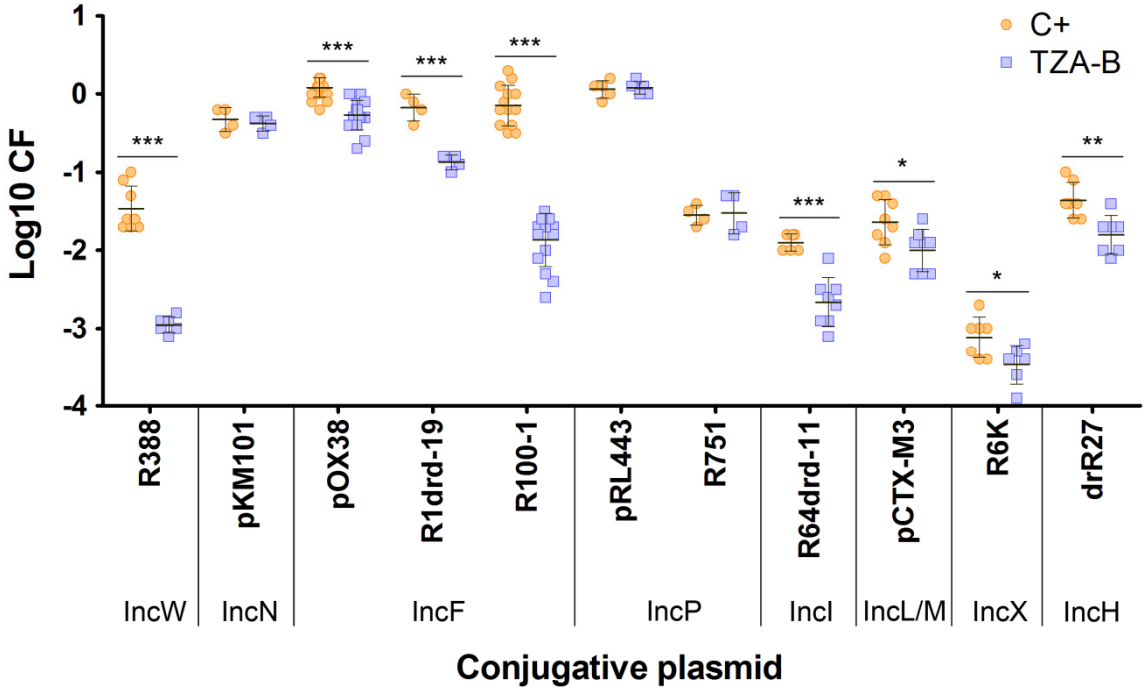



**Fig 4. CF of prototype plasmids in the presence of TZA-B.** CF in the presence of 0.4 mM TZA-B (TZA-B, blue squares) or in its absence (C+, orange circles) using a representative set of conjugative plasmids. Each point represents the result of one independent experiment in logarithmic scale measured by plate-conjugation assay. Horizontal and vertical bars represent the mean ± SD of each group of data (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Inc, incompatibility group (Taylor *et al.* 2004).

### TZA-B inhibits mobilization helped by IncW and IncF plasmids

In addition to conjugative plasmids, mobilizable plasmids are also important carriers of AbR genes. For mobilization, they need the MPF system of a conjugative plasmid present in the donor cell, and even its coupling protein in some cases (ColE1 and RSF1010). To find out which mobilizable plasmids were affected by TZA-B, mobilization of ColE1, RSF1010 and CloDF13 was analyzed in the presence of different helper plasmids. As shown in **Fig 5**, mobilization of plasmids CloDF13 (which encodes its own coupling protein) or ColE1 was affected when the helper plasmid used was itself susceptible to TZA-B (R388, pOX38 or R100-1). On the other hand, mobilization of ColE1 and RSF1010 plasmids helped by the COIN-resistant plasmid pRL443, was unaffected.

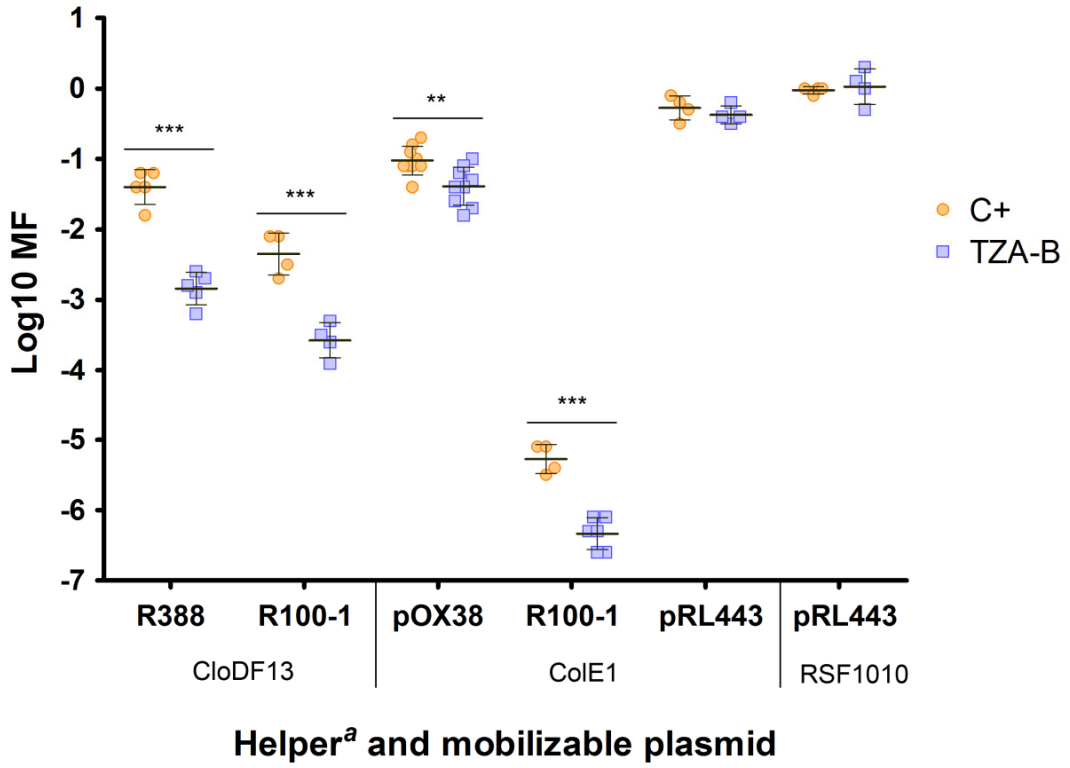



**Fig 5. Mobilization frequency (MF) in the presence of TZA-B.** MF of three mobilizable plasmids in the presence of 0.4 mM TZA-B (TZA-B, blue squares) or in its absence (C+, orange circles), using four different helper plasmids. Each point represents the result of one independent experiment in logarithmic scale measured by plate-conjugation assay. Horizontal and vertical bars represent the mean ± SD of each group of data (** $p < 0.01$, *** $p < 0.001$). [a] CFs of helper plasmids in the presence of mobilizable plasmids were similar to that obtained alone (**Fig 4**).

### Toxicity of natural and chemically synthesized COINs

Toxicity of COINs must be analyzed to select compounds that affect minimally the biodiversity of the targeted ecosystem. It was previously shown that concentrations around the COIN-$IC_{50}$ dose are enough for a compound to prevent the spread of a conjugative plasmid in a bacterial population (Getino *et al.* 2015). Thus, in order to assess the toxicity levels of different COINs, we must determine whether levels below the COIN-$IC_{50}$ exert toxic effects in higher organisms or microbial species. For this purpose, we compared cytotoxic, antibacterial and antifungal activities of the various types of COINs discovered to date, using a variety of human cell lines, bacterial and fungal strains. As shown in **Table 1**, toxicity values (toxic-$IC_{50}$) of all COINs on human cell lines was around 100 μM. Antibacterial and antifungal activities were more variable. Synthetic 2-ODA was bactericidal (toxic-$IC_{90}$) over 7 μM versus *Mycobacterium smegmatis*. 2-HDA was bactericidal versus *M. smegmatis* and fungicidal versus

*Aspergillus nidulans* and *Candida albicans* at similar levels. On the contrary, TZA-A, TZA-B, oleic and linoleic acids showed significantly lower antibacterial and antifungal activities, their toxic-$IC_{90}$ values ranging over 100 µM.

**Table 1. Comparative analysis of COIN toxicity properties.**

| COIN | Toxic-$IC_{50}$ (µM) | | | | | Toxic-$IC_{90}$ (µM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **A549** | **HCT-116** | **PSN1** | **T98G** | **Fibroblasts** | **Saur** | **Msme** | **Anid** | **Calb** |
| **TZA-A** | 60 | 70 | 90 | 90 | 190 | 230 | 230 | > 230 | > 230 |
| **TZA-B** | 90 | 90 | 180 | 180 | 180 | 120 | 120 | > 230 | > 230 |
| **2-HDA** | 40 | 40 | 80 | 100 | 100 | 30 | 8 | 8 | < 4 |
| **2-ODA** | 90 | 70 | 180 | 150 | 90 | 350 | 7 | 220 | 220 |
| **Oleic acid** | 40 | 180 | 80 | 180 | 350 | > 420 | > 420 | > 220 | > 220 |
| **Linoleic acid** | 90 | 180 | 90 | 180 | 300 | > 360 | 360 | > 360 | > 360 |

Toxicity properties of TZA-A and TZA-B compared to previously described COINs (Fernandez-Lopez *et al.* 2005, Getino *et al.* 2015). Inhibitory concentrations for cytotoxic (toxic-$IC_{50}$), antibacterial and antifungal activities (toxic-$IC_{90}$) using different human cell lines, bacteria and fungus species (mean value of three independent experiments). Saur, *Staphylococcus aureus*; Msme, *M. smegmatis*; Anid, *A. nidulans*; Calb, *C. albicans*.

## 4.4. Discussion

COIN application in clinical and environmental settings demands non-toxic, easy to obtain, chemically and biologically stable molecules. COINs discovered to date have limitations that deviate from ideality, such as obtainability, stability or toxicity (Fernandez-Lopez *et al.* 2005, Getino *et al.* 2015). For that reason, a collection of natural compounds extracted from marine microorganisms was screened to find compounds suitable for environmental use. Using AQUAc, a collection of partially purified natural compounds, two new COINs, TZA-A and TZA-B, were discovered (**Fig 3**). Their potency (**Fig 2**) was similar to that of previously identified uFAs (oleic and linoleic acids) (Fernandez-Lopez *et al.* 2005) and of the chemically synthesized 2-HDA (Getino *et al.* 2015).

TZAs are fungal polyketides with chemical structures more complex than previously described COINs (Fernandez-Lopez *et al.* 2005, Getino *et al.* 2015). They are carboxylic acids containing two aromatic rings at the end of an unsaturated aliphatic chain. As a consequence, they belong to the same group as previously reported inhibitors. The

independent isolation of these compounds confirms the essentiality of these two chemical characteristics (a carboxylic group and a long, unsaturated aliphatic chain) for COIN activity. Interestingly, the TZA variant TZA-E, which contains an additional hydroxyl group distal to the carboxylic acid in its chemical structure (**Fig 3A**), was inactive (**Fig 3B**). It thus seems that a substantial hydrophobic moiety is important for COIN function, a characteristic that is functionally broken by a distant single hydroxyl group in the bulky TZA-E.

In addition to potency and structural similarities, the shared spectra of plasmids affected by the action of TZAs and previously analyzed COINS points to a common mechanism of inhibition. IncW and IncF conjugative plasmids, as well as their mobilizable plasmids, represent the main targets of the COINs described here (**Figs 4 and 5**) as well as in previous publications (Fernandez-Lopez *et al.* 2005, Getino *et al.* 2015). These results suggest a shared target in conjugation and mobilization, probably being part of the MPF system of affected conjugative plasmids, also used for transfer of mobilizable plasmids.

TZAs were previously reported to inhibit superoxide anion production (Kuramoto *et al.* 1997, Malmstrom *et al.* 2000), nitric oxide production and protein tyrosine phosphatase 1B activity in inflammatory cells (Quang *et al.* 2014). In addition, two recent studies analyzed antimicrobial and cytotoxic effects of these fungal polyketides. TZA-A was found to inhibit conidial germination of the rice blast fungus *Magnaporthe oryzae* (toxic-$IC_{50}$ = 37 μM), and showed weak activity against the Gram-positive bacteria *Brevibacillus brevis*, the fungi *Mucor miehei* and *Paecilomyces variotii*, and HeLaS3 cells at a concentration of 185 μM. Germination of the grey mold *Botrytis cinerea* and the potato blight caused by the oomycete *Phytophthora infestans* were not affected at comparable concentrations (Sandjo *et al.* 2014). In an independent work (Cardoso-Martinez *et al.* 2015), the antimicrobial activity of TZA-B against *S. aureus*, *Salmonella sp.*, *Klebsiella pneumoniae*, *E. coli*, *Bacillus cereus*, *Proteus mirabilis*, *Enterococcus faecalis*, and *C. albicans* showed no effect below 364 μM COIN concentration. Moreover, leukemic and lymphoblastic cell lines (K562, U937, Jurkat and Raji) showed no response at 100 μM. These data, together with our results (**Table 1**), situate the TZAs, along with the previously identified oleic and linolenic acids (Fernandez-Lopez *et al.* 2005), as the least toxic COINS identified so far. A comparison of toxicity values with the COIN potency of the different molecules tested, revealed that TZA-A, TZA-B, oleic and linoleic acid presented toxic-$IC_{50}$ levels that were above their COIN threshold (COIN-$IC_{50}$ ≈ 50 μM) (**Fig 2**) (Fernandez-Lopez *et al.* 2005). 2-HDA and 2-ODA were non-toxic at COIN concentrations in almost all human cell lines tested (COIN-$IC_{50}$ ≈ 50 μM) (Getino *et al.* 2015), but exerted strong toxic effects in

mycobacterial and/or fungal species (**Table 1**). Although TZA-B showed COIN activity at non-toxic concentrations, cytotoxic and COIN thresholds were too close. A key finding from this work is that toxicity and COIN activity do not necessarily correlate with each other, since TZA-B and 2-HDA presented similar COIN-$IC_{50}$ concentrations, yet the later was more toxic to bacterial and fungal strains. This opens the possibility of further screening natural and synthetic derivatives with lower toxicity and enhanced COIN activity.

In summary, the COINs reported here and in previous work provide important ammunition in the search for optimal COINs. Their different characteristics make them applicable to different purposes. On the one hand, 2-HDA and 2-ODA are easily obtainable by chemical synthesis (Tasdemir *et al.* 2010, Carballeira *et al.* 2012, Sanabria-Rios *et al.* 2014) and have provided important structural information (Getino *et al.* 2015). Nevertheless, their antifungal (Gershon and Shanks 1978, Carballeira *et al.* 2006), antiprotozoal (Tasdemir *et al.* 2010, Carballeira *et al.* 2012), antimicrobial and cytotoxic activities (Konthikamee *et al.* 1982, Morbidoni *et al.* 2006, Sanabria-Rios *et al.* 2014), exclude their use in natural environments, where biodiversity must be maintained, and confine their use to academic setups. On the other hand, TZAs A and B (this work), as well as oleic and linoleic acids (Fernandez-Lopez *et al.* 2005), are potentially more unstable, but they are natural compounds with reduced toxicity (**Table 1**), some of them being normal constituents of the human diet (Di Vaio *et al.* 2013). This makes them potential COINs for their use in natural environments, either in combination with effective antioxidants or through delivery vehicles with a protective atmosphere. In general, COINs show a shared and relatively broad range of affected plasmids, among them IncF plasmids, the most common AbR carriers in pathogenic *Enterobacteriaceae* (Carattoli 2009). Furthermore, non-toxic COINs could be used in ecological reservoirs of AbR genes, or as a combination treatment with antibiotics to prolong their useful lifetime, or even as virulence inhibitors for pathogens such as *Legionella, Helicobacter, Neisseria, Brucella* or *Bartonella*, which use secretion systems similar to conjugative systems.

## 4.5. Materials and Methods

**Bacterial strains and plasmids.** Derivatives of *E. coli* strain DH5α (Grant *et al.* 1990) containing either the conjugative plasmid pJC01 (Getino *et al.* 2015) or plasmids pSU2007::Tn*lux* and pUC18::*lacI*$^{q}$ (Fernandez-Lopez *et al.* 2005) were used as donor strains in fluorescence-based or luminescence-based HTC experiments, respectively. Rifampicin-resistant derivative *E. coli* CSH53 (Miller *et al.* 1970) was used as recipient strain in luminescence-based HTC assay and as pSU2007::Tn*lux* containing strain in

control assays (Fernandez-Lopez *et al.* 2005). Streptomycin-resistant derivative *E. coli* BL21 (DE3) (Studier and Moffatt 1986) was used as recipient strain expressing T7 RNA polymerase in fluorescence-based HTC assay (Getino *et al.* 2015). *E. coli* DH5α (Grant *et al.* 1990) containing different conjugative and mobilizable plasmids (**S2 Table**) and a rifampicin-resistant derivative of *E. coli* MDS52 (Posfai *et al.* 2006) were used as donor and recipient strains respectively in plate-conjugation assays.

**Reagents.** When appropriate, antibiotics (Apollo) were added at the following concentrations: ampicillin sodium salt (Ap; 100 μg/ml), chloramphenicol (Cm; 25 μg/ml), nalidixic acid (Nx; 20 μg/ml), rifampicin (Rif; 50 μg/ml), streptomycin (Sm; 300 μg/ml), tetracycline (Tc; 10 μg/ml) and trimethoprim (Tp; 10 μg/ml). Oleic and linoleic acids (Sigma-Aldrich) were used as control COINs, DMSO (Sigma-Aldrich) was used as solvent and IPTG (Sigma-Aldrich) as T7 RNA polymerase inductor. Bacterial cultures were set up in LB-broth and LB-agar (Pronadisa). M9 broth (Sigma-Aldrich) were used to resuspend bacteria after mating and perform serial dilutions.

**Isolation of TZA-B.** TZA-B producer *Penicillium* sp. strain CECT 20935, isolated from a *Porifera* sp. collected in Guatemala and grown in potato dextrose agar plates (Pronadisa), was used to inoculate 40 ml of potato dextrose broth (Pronadisa). This first inoculum was grown for 3 days at 24 ºC and 200 rpm. Then, 15 ml were added to 250 ml of the same media and cultured for 7 days at 24 ºC and 200 rpm. Fermentation broth (4 l) was filtered off with dicalite® (Dicalite Europe) and the mycelial cake was extracted twice by adding 1.5 l of a mixture of EtOAc/MeOH 3:1 and soaking for 1 h. The organic solvent was filtered off and the pellet dried under reduced pressure. Dried extracts (2.8 g) were fractionated by vacuum flash chromatography using a stepwise gradient of Hexane/EtOAc/MeOH. Fractions containing TZA-B (eluted with Hexane/EtOAc 2:8) were applied to a silica gel column and flash-chromatographed by elution with a Hexane/EtOAc gradient. Fractions eluted with Hexane/EtOAc 75:25, afforded 105 mg of 93 % pure TZA-B.

**Structural elucidation of TZA-B.** TZA-B has a maximum UV absorption at 300 nm. The molecular formula was determined to be $C_{18}H_{26}O_2$ based on the MS (*m/z* 274.3) and NMR spectral data. Extensive NMR experiments ($^1$H NMR, $^{13}$CNMR, $^1$H-$^1$H COSY, gHSQC, gHMBC and NOESY) indicate that TZA-B has three methyl groups, two methylenes, twelve methines (six of them olefinic), one quaternary carbon and one exchangeable proton. These data were identical with those for TZA-B, previously reported in the literature (Kuramoto *et al.* 1997). $^1$H-NMR and $^{13}$C-NMR data (**Fig 1**) were recorded on a Varian "Mercury 400" spectrometer (Agilent Technologies) at 400 and 100 MHz, respectively. gHMQC and gHMBC experiments were carried out using an inverse

resonance probe. Chemical shifts are reported in ppm relative to solvent ($CDCl_3$ $\delta_H$ 7.24, $\delta_C$ 77.0). MS data were recorded on an Agilent/HP 1100 Series Simple Quad Mass Spectrometer (Agilent Technologies), using both, ESI (+) y (-) and APCI (+) y (-) ionization sources.

**HTC screening.** A luminescence-based HTC assay was performed as previously described (Fernandez-Lopez *et al.* 2005). Briefly, a *lux* operon under the control of a *lac* promoter encoded by the R388 derivative pSU2007::Tn*lux* is repressed in donor cells by the $LacI^q$ repressor carried in the co-resident non-mobilizable multi-copy plasmid pUC18::*lacI*$^q$. Upon conjugation, pSU2007::Tn*lux* but not pUC18::*lacI*$^q$ is transferred to recipient cells, where light is produced. Absolute luminescence emitted by transconjugant cells was then measured and normalized to the mean value of the corresponding plate. Control assays to discard non-specific compounds were carried out by growing a pSU2007::Tn*lux* containing strain without plasmid pUC18::*lacI*$^q$ and measuring light production. Similarly, HTC assay based on the emission of fluorescence employed plasmid pJC01 as test plasmid (Getino *et al.* 2015). In donor cells, the *gfp* gene present in this R388 derivative is not expressed, due to the inactivity of its T7 promoter. When pJC01 plasmid is transferred to the recipient strain, which carries T7 RNA polymerase, GFP is produced. CF was estimated as the ratio of absolute fluorescence emitted by transconjugant cells and $OD_{600}$ as a measurement of the total number of cells. Relative CF in the presence of a compound was thus determined as a fraction of the CF in the absence of it (adding the same volume of solvent).

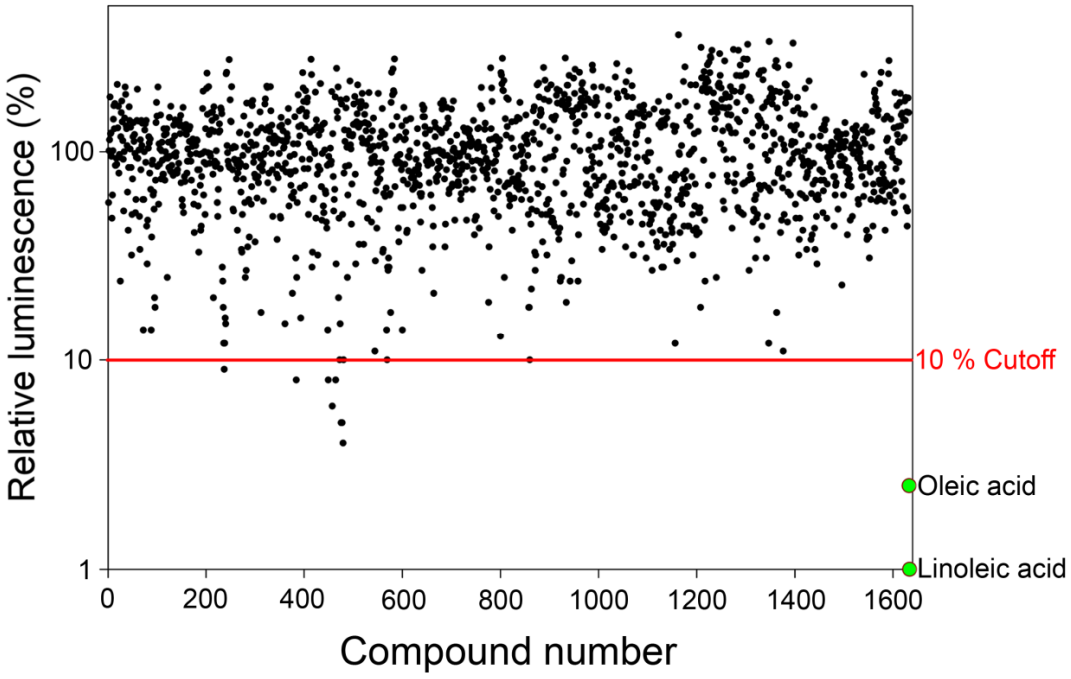
**Plate-conjugation assay.** For the plate-mating procedure, a 200 µl mixture of equal volumes of donor and recipient cultures previously washed, both in stationary phase, was centrifuged and resuspended in 15 µl LB-broth. 5 µl of this mixture were placed on top of 96-well microtiter plate wells containing 150 µl LB-agar (± COINs) and conjugation was allowed to proceed, in general, for 1 h at 37 ºC. The temperature-sensitive IncH plasmid drR27 was allowed to conjugate for 2 h at 25 ºC (Taylor and Levine 1980). Bacteria were then resuspended in 150 µl M9 broth and corresponding dilutions were plated on selective media. CF was calculated as the number of transconjugant cells per donor, whereas MF was calculated as the number of cells receiving the mobilizable plasmid per donor. Since this type of frequency data were log-normally distributed, means are calculated using decimal logarithms of data. Relative CF or MF in the presence of a compound was determined as a fraction of the CF of MF in the absence of it (adding the same volume of solvent).

**Toxicity assays.** Cell culture cytotoxicity assays were performed as described (Mosmann 1983, Denizot and Lang 1986) using human foreskin fibroblasts ATCC SCRC-

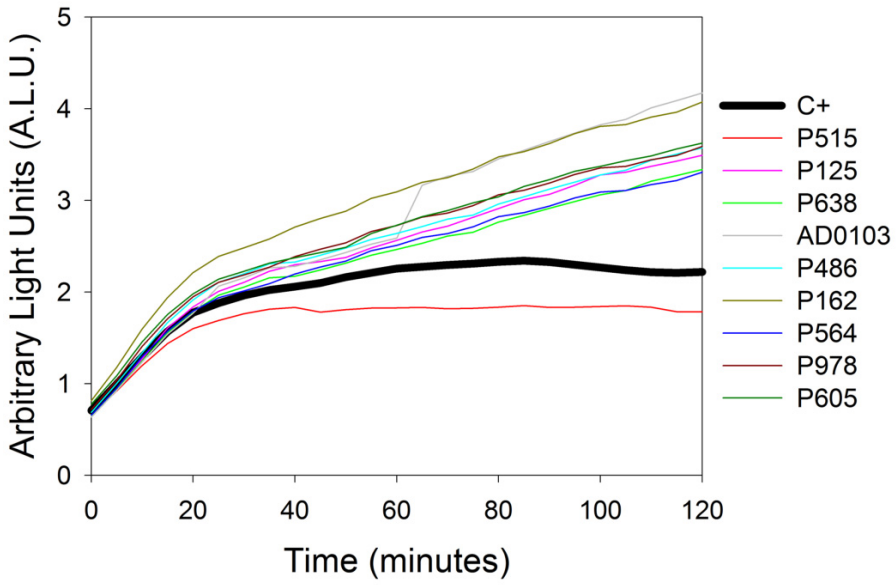
1041 (Amit *et al.* 2003), lung carcinoma cells A549 (Lieber *et al.* 1976), colorectal carcinoma cells HCT-116 (Reske-Kunz and Rude 1984), pancreatic adenocarcinoma cells PNS1 (Verovski *et al.* 1996) or glioblastoma multiforme cells T98G (Stein 1979). Antibacterial activity was determined using a conventional microtiter broth-dilution technique (Wiegand *et al.* 2008) for two reference strains, *S. aureus* CECT 794 and *M. smegmatis* DSMZ 43756. Antifungal activity was measured using the reference method antifungal broth dilution susceptibility test (National Committee for Clinical Laboratory Standars) against two species: *A. nidulans* (Microorganisms Collection of Biomar Microbial Technologies) and *C. albicans* CECT 1394.

**Statistical analysis.** Mean comparison between two different conditions was carried out by using t test tool from GraphPad Prism® (v 5.0) biostatistics software (San Diego, CA).

### 4.6. Supplemental figures




**S1 Fig. Point cloud representation obtained from AQUAc HTC screening.** Absolute luminescence emitted by transconjugant cells was measured in arbitrary light units (A. L. U.) and normalized to the mean value of the corresponding plate (100 %). Each point represents the mean of two independent experiments obtained by luminescence-based HTC assay in the presence of bactericidal or non-bactericidal compounds (220 ng/ml or 11 μg/ml, respectively). A relative luminescence cutoff of 10 % was arbitrarily established (red) to select the most active compounds. Oleic and linoleic acids (green) were used at 1 mM concentration as control COINs.




**S2 Fig. Kinetic luminescence assay of selected hits from AQUAc screening.** *E. coli* CSH53 containing pSU2007::Tn*lux* (but not pUC18::*lacI*$^{q}$) was cultured overnight, diluted until $OD_{600}$ = 0.1 and grown for 2 h in the absence (C+) or the presence of each potential inhibitor (50 µg/ml). The figure shows the kinetics of light emission, measured every 5 min and represented over time.

## 4.7. Supplemental tables

**S1 Table. Potency of AQUAc selected hits.**

| Hit | CF (%) | | | |
|---|---|---|---|---|
| | **5 µg/ml** | **10 µg/ml** | **25 µg/ml** | **50 µg/ml** |
| **P515** | 1 | 1 | 1 | 1 |
| **P125** | 55 | 22 | 15 | 8 |
| **P638** | 94 | 75 | 28 | 25 |
| **AD0103** | 109 | 91 | 42 | 19 |
| **P486** | - | 17 | 26 | 7 |
| **P162** | 17 | 23 | 23 | 6 |
| **P564** | 110 | 83 | 98 | 22 |
| **P978** | 22 | 26 | 23 | 8 |
| **P605** | 5 | 6 | 6 | 2 |

CF in the presence of selected hits from AQUAc screening. Absolute luminescence emitted by transconjugant cells was measured in A.L.U. and relativized to the control in the absence of added COINs (100 %). Each value represents the mean of two independent experiments obtained by luminescence-based HTC assay in the presence of the given concentrations of selected hits. The hyphen represents no data for that point.

**S2 Table. Conjugative and mobilizable plasmids used.**

| Plasmid | Inc[a] | MOB[b] | MPF[c] | Reference |
|---|---|---|---|---|
| **R388** | W | F11 | T | (Datta and Hedges 1972) |
| **pKM101** | N | F11 | T | (Langer *et al.* 1981) |
| **pOX38** | FI | F12 | F | (Chandler and Galas 1983) |
| **R1drd-19** | FII | F12 | F | (Meynell and Datta 1967) |
| **R100-1** | FII | F12 | F | (Yoshioka *et al.* 1987) |
| **pRL443** | P1α | P11 | T | (Elhai *et al.* 1997) |
| **R751** | P1β | P11 | T | (Thorsted *et al.* 1998) |
| **R64drd-11** | I1α | P12 | I | (Komano *et al.* 1990) |
| **pCTX-M3** | L/M | P131 | I | (Golebiewski *et al.* 2007) |
| **R6K** | X2 | P3 | T | (Kolter and Helinski 1978) |
| **drR27** | HI1 | H11 | F | (Whelan *et al.* 1994) |
| **CloDF13** | ColE | C11 | - | (van Putten *et al.* 1987) |
| **ColE1** | ColE | P5 | - | (van Rensburg and Hugo 1969) |
| **RSF1010** | Q1 | Q11 | - | (Derbyshire *et al.* 1987) |

[a] Inc, incompatibility group (Taylor *et al.* 2004). [b] MOB, MOB group (Garcillan-Barcia *et al.* 2009). [c] MPF, mating pair formation type (Guglielmini *et al.* 2011). The hyphen represents the absence of MPF in mobilizable plasmids.

# Global discussion

Antibiotic resistance (AbR) dissemination has become a worldwide crisis (WHO 2014). Since conjugative plasmids are the main carriers involved in this dissemination (Norman *et al.* 2009), strategies to control plasmid conjugation have been proposed as potential solutions (Baquero *et al.* 2011). On the one hand, natural mechanisms that bacteria employ as defense barriers against invading genomes, such as restriction-modification (RM) or CRISPR-Cas systems (Wilkins 2002, Marraffini and Sontheimer 2008), could be exploited to control horizontal gene transfer (HGT). Conjugative plasmids themselves display mechanisms to minimize their associated burden or compete with related or unrelated plasmids. Thus, FinOP systems aid plasmids to regulate their own transfer (Frost and Koraimann 2010), exclusions systems avoid conjugation of related plasmids to the same recipient bacteria (Garcillan-Barcia and de la Cruz 2008), and fertility inhibition systems block conjugation of unrelated plasmids from the same donor cell (Gasson and Willetts 1975). On the other hand, artificial strategies have been designed for the same purpose. For instance, intrabodies against R388 relaxase expressed in recipient cells inhibited R388 conjugation (Garcillan-Barcia *et al.* 2007) and pIII protein of bacteriophage M13 inhibited F conjugation by obstructing conjugative pilus (Lin *et al.* 2011). In this context, the main aim of this thesis was to find new mechanisms for controlling bacterial conjugation, and thus fighting AbR propagation. For doing that, three main approaches were carried out. First, searching for *E. coli* functions involved in conjugation that could act as targets or barriers to control conjugative transfer. Second, exploring the interactions between unrelated conjugative plasmids in donor bacteria to find new fertility inhibition systems. And finally, screening for compounds that function as conjugation inhibitors (COINs).

In a previous work, a collection of *Escherichia coli* mutants was tested as recipients of plasmid R388 (Perez-Mendoza and de la Cruz 2009). No non-essential functions were required for surface mating, although some mutations affecting lipopolysaccharide (LPS) biosynthesis had drastic effects in F liquid conjugation, enhancing the importance of this membrane constituent in mating pair stabilization. To complete the study, the Keio collection of deletion mutants of non-essential *E. coli* genes (Baba *et al.* 2006) was analyzed as donors of R388, a broad host range (BHR) plasmid with host-encoded conjugative mechanisms theoretically more conserved. This feature would allow the identification of potential conjugation targets present in a broad range of bacteria. However, no donor functions were found essential for R388 conjugation that could act as targets for control of bacterial conjugation. Some mutations associated with membrane composition or ATP synthesis reduced R388 conjugation by 10-fold. These results suggested the participation of donor membrane composition and energy supply in conjugation efficiency. Accordingly, systematic screenings for recipient genes involved in conjugation of R388 and ICE*Bs1* also pointed to membrane-related functions (Perez-

Mendoza and de la Cruz 2009, Johnson and Grossman 2014), which might be affecting donor-recipient contact. In fact, recipient LPS was identified as the specific receptor for the PilV adhesin of IncI plasmid R46 during liquid conjugation (Ishiwa and Komano 2000) and the outer membrane protein OmpA was reported to interact to F plasmid TraN for mating pair stabilization (Klimke *et al.* 2005). In addition, a deficient ATP supply in donor cells caused by deletions in different ATP synthase subunits could inhibit the activity of the ATPases required for plasmid conjugation (Chandran Darbari and Waksman 2015).

In general, no host *E. coli* genes were found to be essential for conjugation of the BHR plasmid R388. If any, they might be necessary for bacterial growth or there might be alternative genes with redundant function. As an example, the essential enzyme DNA polymerase III is involved in synthesis of plasmid complementary strand during conjugation both in donors and recipients (Wilkins and Hollom 1974, Kingsman and Willetts 1978). This and other essential functions, more evolutionarily conserved than non-essential genes (Jordan *et al.* 2002), could be required for conjugative transfer of BHR plasmids like R388. Essential functions for bacterial growth are targets of common antibiotics. These compounds exert high selective pressures on bacteria, promoting the emergence of AbR. In contrast, COINs should allow bacterial growth, thus avoiding the rapid appearance of resistant variants collateral to common antibiotic therapies. For that reason, essential functions are not desirable targets to inhibit bacterial conjugation.

Mutants inhibiting conjugation can provide host functions potentially useful as targets to control conjugation. Similarly, an improvement in conjugation efficiency could be caused by deletion of potential conjugation barriers. The study of the Keio collection did not allow the identification of any mutant improving R388 conjugation, either as donors or recipients. Therefore, *E. coli* DH5α contains no non-essential host genes negatively affecting R388 conjugation. Since *E. coli* lab strains act as proficient recipients of R388 transfer, the ability of clinically isolated enterobacteria to block the entry this plasmid was tested. As a result, most clinical isolates analyzed contained barriers against conjugation. Few of them were identified in resident plasmids, which encoded genes for bacteriocin production able to kill susceptible donor cells, thus indirectly inhibiting conjugation. Other barriers were encoded by the bacterial chromosome. An *E. coli* strain isolated from a urinary tract infection was selected as an example (Ejrnaes *et al.* 2011). Its inhibition mechanism affected conjugation of different plasmids, as well as transformation of plasmids extracted from *E. coli* lab strains. When these plasmids were extracted from the selected recipient, transformation efficiency was restored. These results indicated the presence of a RM system in the selected recipient. While the smallest plasmid was not affected due to the absence of restriction sites, larger plasmids

extracted from lab strains were inhibited due to the presence of more restriction sites, favoring digestion by recipient endonucleases. In addition, affected plasmids extracted from the selected recipient were protected from digestion by methylation of restriction sites, a process normally used for protection of its own chromosome. As in this case, most prokaryotic chromosomes encode RM systems as defense barriers against potentially harmful invading genomes (Oliveira *et al.* 2014). In fact, conjugative plasmids have evolved to evade these systems through anti-restriction proteins, such as the ssDNA protector ArdC of IncW plasmids (Belogurov *et al.* 2000). Therefore, RM systems are important bacterial barriers to prevent HGT with high potential in the fight against AbR dissemination.

Conjugative plasmids also display mechanisms to regulate their own transfer or inhibit conjugation of other plasmids. Among them, fertility inhibition systems are promising tools to stop AbR propagation. They inhibit conjugation of unrelated plasmids present in the same donor bacteria, probably to compete for colonization of new hosts (Gasson and Willetts 1975). For example, FinC protein of the mobilizable plasmid CloDF13 is thought to inhibit the coupling protein of its helper in favor of its own mobilization (Willetts 1980). Several groups of plasmids are targeted by fertility inhibition factors encoded by different conjugation systems. Transfer of IncF plasmids is targeted by five known genes (*finQ*, *finW*, *finC*, *finU*, and *finV*) (Gasson and Willetts 1975), IncW plasmids are inhibited by *fiwA* and *fiwB* (Fong and Stanisich 1989), IncP plasmids by *fipA* and *pifC* (Winans and Walker 1985, Miller *et al.* 1985), and pTi plasmid by *osa* (Close and Kado 1991). The best described is Osa, encoded by IncW plasmids. Recently, T-DNA degradation by Osa DNase activity was observed, a mechanism likely responsible of its mode of inhibition (Maindola *et al.* 2014). In addition, the inhibition of pTi transfer by Osa homologs ICE1056Fin and FiwA, and even unrelated factors FipA and PifC, suggested a common mode of action among different fertility inhibition systems. This feature places fertility inhibition as a conserved mechanism useful for preventing the propagation of a broad range of conjugative systems.

In order to add more knowledge to this undeveloped field, the interaction network between unrelated plasmids should be defined. Specifically, this work focused on BHR conjugative plasmids, capable of carrying resistance genes to distantly related bacteria (Mazodier and Davies 1991). The study of interactions between IncW systems and a representative set of conjugative plasmids in donor bacteria revealed two novel fertility inhibition systems affecting IncW conjugation, encoded by IncFI plasmid pOX38 and IncI1 plasmid R64. Interestingly, both plasmids shared the replication protein RepC/PifC (Tanimoto and Iino 1984), responsible of IncP inhibition caused by F plasmid (Miller *et al.* 1985) and probably by R64 (Datta *et al.* 1971). Accordingly, this gene is absent from

IncFII plasmids R1drd19 and R100-1, which had no effect in R388 conjugation. In addition, when R388 was substituted with a reduced synthetic version, the inhibitory effect caused by pOX38 and R64 was lower than wild type in both cases, suggesting a common mechanism of action. However, no effect was previously observed on R388 conjugation in the presence of FipA (pKM101) or PifC (F) (Santini and Stanisich 1998). These proteins were responsible of inhibiting the coupling protein of IncP plasmids (Santini and Stanisich 1998). Similarly, the R388 inhibition system encoded by pOX38 could target R388 coupling protein TrwB. Nevertheless, TrwB overexpression in donor cells only improved R388 6-fold in the presence of pOX38. Besides R64 and F (encoding PifC), other groups of plasmids were capable of reducing both IncP and IncW plasmids fertility. This was the case of IncX2 plasmid R6K, IncN plasmids (encoding FipA), and even IncP (encoding FiwA/FiwB) and IncW plasmids were reciprocally repressed (Olsen and Shipley 1975, Coetzee *et al.* 1972). This inhibitory network suggests that fertility inhibition could be broadly distributed among conjugative plasmids to compete for invading bacterial populations, probably presenting a common mechanism of action related to DNA degradation (Maindola *et al.* 2014).

Bacterial conjugation can be artificially controlled by molecules interfering with specific components of the process, such as the relaxase (Garcillan-Barcia *et al.* 2007) or the conjugative pilus (Lin *et al.* 2011). Moreover, COINs can be found by screening a collection of compounds for their ability to reduce conjugation in whole cells. Thus, a derivative of plasmid R388, able to produce luminescence after its arrival to recipient cells, was used to analyze COIN activity of 12,000 natural compounds from the NatChem library (Fernandez-Lopez *et al.* 2005). Unsaturated fatty acids (uFAs) oleic and linoleic acids inhibited conjugation of IncW and IncF plasmids, without affecting bacterial growth. The most potent COIN identified was dehydrocrepenynic acid (DHCA), a triple-bonded fatty acid extracted from tropical plant seeds (Gussoni *et al.* 1994).

In order to chemical and biologically characterize the observed COIN activity, a set of 2-alkynoic fatty acids (2-AFAs) was synthesized. 2-hexadecynoic acid (2-HDA) was identified as the most effective synthetic COIN, with similar potency than natural uFAs (Fernandez-Lopez *et al.* 2005). Analysis of 2-HDA analogs confirmed the importance of a carboxylic group and of the presence of at least one unsaturation. Other features, such as carbon chain length or triple bond position, influenced as well 2-AFA effectiveness. A clinically representative set of conjugative plasmids was tested in the presence of 2-HDA to find out its spectra of action. As natural uFAs (Fernandez-Lopez *et al.* 2005), 2-HDA also inhibited IncF plasmids, the most common carriers of AbR genes in pathogenic Enterobacteriaceae (Carattoli 2009). Besides IncW and IncF, IncH plasmids were also affected by 2-HDA. While IncI, IncX, and IncL/M plasmids were moderately inhibited,

IncN and IncP plasmids were completely resistant to COIN action, even at high concentrations. Interestingly, mobilization helped by affected conjugative plasmids was also inhibited, suggesting a shared target in mating pair formation (MPF) system. Also interesting for future applications is the fact that conjugation was inhibited irrespective of the bacterial host used as donor, the conjugative partner where COIN effect took place. The most remarkable result was obtained through a liquid mating experiment using the multi-resistant plasmid R1drd19. In the absence of COINs, the IncF plasmid was able to invade the bacterial population after just four generations, due to its high infectivity. However, the presence of 2-HDA in the mating medium not only blocked plasmid invasiveness, but also reduced the prevalence of plasmid-containing cells. This result indicated that plasmid conjugation was reduced in favor of plasmid burden, which affected growth rate of plasmid-containing cells, favoring growth of plasmid-free cells. The reversion from plasmid invasion to plasmid loss occurred at 2-HDA concentrations from 50 μM, comparable to the observed $IC_{50}$ in R388 inhibition. This suggested that an inhibition of conjugation of 50 % was sufficient to prevent plasmid spread in the absence of selective pressure. These observations highlighted the potential application of COINs to prevent AbR dissemination.

Since some proteins associated with bacterial membranes were inhibited by uFAs, such as the replication ATPase DnaA (Yung and Kornberg 1988), a recent work analyzed the activity of conjugative ATPases in the presence of uFAs (Ripoll-Rozada *et al.* 2016). The component of R388 MPF system TrwD, involved in pilus synthesis and DNA translocation (Kerr and Christie 2010, Atmakuri *et al.* 2004), was identified as the potential target of these COINs in R388. Conjugation experiments correlated perfectly with TrwD ATPase activity in the presence of different compounds, including saturated fatty acids, uFAs (Fernandez-Lopez *et al.* 2005), synthetic AFAs, and AFAs inactive analogs. In addition, the non-competitive inhibition observed suggested that COIN binding site was different from nucleotide binding site. Specifically, docking approaches indicated a putative binding site in a pocket comprised by the N-terminal domain and the linker region of TrwD. It is possible that the obstruction of this region limits protein movements needed for ATPase activity (Ripoll-Rozada *et al.* 2016).

Although triple-bonded fatty acid 2-HDA is a promising COIN without antibacterial activity against *E. coli* (Carballeira *et al.* 2012), it presented toxicity against fungi (Gershon and Shanks 1978, Carballeira *et al.* 2006), protozoa (Tasdemir *et al.* 2010, Carballeira *et al.* 2012), Gram positive bacteria, some Gram-negative bacteria, and eukaryotic cells (Konthikamee *et al.* 1982, Morbidoni *et al.* 2006, Sanabria-Rios *et al.* 2014). These problems of synthetic 2-AFAs exclude their application from environmental settings, where biodiversity must be maintained. On the other hand, natural uFAs like

oleic and linoleic acids present a stability drawback, since they have double bonds susceptible to oxidation (Niki *et al.* 2005). The other natural uFA with COIN activity, DHCA, was not easily obtainable (Gussoni *et al.* 1994). In search of suitable COINs for environmental use, a collection of bioactive compounds isolated from marine microorganisms was analyzed. Consequently, tanzawaic acids (TZAs) A and B were found to reduce R388 conjugation without affecting bacterial growth. TZAs are fungal polyketides more complex than previous COINs but they are carboxylic acids too, with two aromatic rings at the end of an unsaturated aliphatic chain, which confirmed the importance of these two chemical characteristics for COIN activity. The inactivity of TZA-E, which contains an additional hydroxyl group distal to the carboxylic group, suggested that a substantial hydrophobic moiety was important for COIN activity as well. Potency and spectra of plasmids affected were similar to previous identified COINs, pointing to a common mechanism of action. TZAs were reported to inhibit superoxide anion production (Kuramoto *et al.* 1997, Malmstrom *et al.* 2000), nitric oxide production, and protein tyrosine phosphatase 1B activity in inflammatory cells (Quang *et al.* 2014). In addition, recent studies analyzed antimicrobial and cytotoxic effects of these fungal polyketides (Sandjo *et al.* 2014, Cardoso-Martinez *et al.* 2015). Toxicity properties of COINs discovered to date, including uFAs, 2-AFAs, and TZAs, were tested against different human cell lines, bacteria, and fungi. In accordance with published data, natural COINs (uFAs and TZAs) presented reduced toxicity compared to synthetic 2-AFAs. TZA-A, TZA-B, oleic acid, and linoleic acid were non-toxic at concentrations lower than 50 μM, the minimal COIN concentration needed for preventing plasmid spread in a bacterial population. In contrast, 2-AFAs exerted strong toxic effects in mycobacterial and fungal species tested. Although the promising 2-HDA is easily synthesized (Tasdemir *et al.* 2010) and potentially more stable than uFAs, its toxicity issues confine its use to academic setups. On the contrary, natural uFAs present reduced toxicity, some of them being normal constituents of the human diet (Di Vaio *et al.* 2013). Therefore, natural COINs, particularly oleic and linoleic acids, could be suitable compounds for preventing plasmid conjugation in natural environments.

In summary, *E. coli* DH5α contains no non-essential genes involved in R388 conjugation that could be used as targets or barriers to control conjugation. In contrast, most clinically isolated enterobacteria present mechanisms that prevent the entry of conjugative plasmids, such as bacteriocin production or RM systems. In this sense, more barriers could be found by studying in more detail the mechanisms responsible of controlling the entry of R388 conjugation. Additionally, screening other collections of natural isolates or mutants could provide more information about host mechanisms involved in conjugation of different plasmids. The most prevalent host mechanisms used by bacteria as defense against incoming genomes are RM and CRISPR-Cas systems. RM

systems are "innate immune systems" present in 69 % of prokaryotic genomes (Oliveira *et al.* 2014). They produce significant effects in conjugation, since many plasmids encode anti-restriction proteins to evade these systems (Wilkins 2002). CRISPR-Cas systems target specific sequences of invading genomes, being considered as "adaptive immune systems". CRISPR loci, present in 45 % of bacterial and 84 % of archaeal sequenced genomes (Grissa *et al.* 2007), can be re-designed to target specific sequences of interest. Therefore, this robust tool has countless applications, including transfer inhibition by targeting plasmid DNA (Marraffini and Sontheimer 2008), plasmid loss, or even death of multidrug resistant bacteria by targeting AbR genes (Garneau *et al.* 2010, Bikard *et al.* 2014, Yosef *et al.* 2015).

Inhibition systems encoded by conjugative plasmids can also be considered natural barriers for resistance dissemination. Studying the interactions of IncW systems with unrelated plasmids in donor bacteria, two novel fertility inhibition systems were found in IncFI and IncI1 plasmids. Mutagenesis by transposition of these plasmids is being carried out to find variants unable to inhibit R388 fertility. A potential responsible gene is being cloned to study its effect on R388 conjugation. Additionally, R388 mutants escaping fertility inhibition will be sequenced to look for putative targets of each inhibition system. Once responsible genes or targeted functions are elucidated, their application for controlling bacterial conjugation will be one step closer. An example of a potential application of these fertility inhibition factors would be a plasmid or phage vector designed to carry many fertility inhibition genes, infect plasmid containing cells, and inhibit conjugation of targeted plasmids.

In addition to barriers that bacteria naturally impose, artificial tools were developed to prevent plasmid conjugation. High-throughput screening assays to measure plasmid transfer allowed the identification of two novel sets of COINs, synthetic 2-AFAs and natural TZAs. Characterization of these compounds and their analogs confirmed the importance of a carboxylic group and an unsaturated hydrocarbon chain for COIN activity. IncW and IncF conjugative plasmids, including plasmids mobilized by them, were mainly affected. Interestingly, conjugation inhibition was demonstrated for a wide series of bacterial hosts, underscoring the applicability of this technology in natural environments. Importantly, 2-HDA effect on R1drd19 liquid conjugation was sufficient to reduce the prevalence of plasmid-containing bacteria in the absence of selective pressure. Toxicity analysis against bacteria, fungi, and eukaryotic cells placed natural COINs as suitable compounds for environmental use. As a continuation of this last part of the work, the precise mechanism of inhibition of discovered COINs is being studied. Preliminary data of their effect on plasmid-mediated biofilm formation, plasmid burden, and phage infection point to a deficient pilus synthesis. Electron microscopy analysis of

treated bacteria would confirm this hypothesis. Affected pilus formation agrees with TrwD as the specific MPF target of R388 inhibition (Ripoll-Rozada *et al.* 2016), an enzyme needed for pilus synthesis and DNA translocation (Kerr and Christie 2010, Atmakuri *et al.* 2004). However, many questions remain unanswered. For example, what is the target in IncF plasmids or why IncN and IncP plasmids are resistant to COIN activity? ATPase activity studies of TrwD homologs or other potential targets in these conjugative systems might reveal useful information to answer these questions. The study of the mechanism of inhibition is important for the rational design of molecules with better properties for their application in natural environments. Although presently known COINs display lower potency than natural barriers (restriction or fertility inhibition systems), the application of small molecules in natural environments is easier than proteins or more complex mechanisms. Additionally, the inhibitory effect of COINs was sufficient to displace a conjugative plasmid from a bacterial population. For these reasons, linoleic acid was selected as the most suitable non-toxic COIN, already present in the human diet (Di Vaio *et al.* 2013), to test its effect in natural ecosystems. Zebra fish and mouse guts are the initial microcosms where conjugation in the presence of linoleic acid are being analyzed. Then, more complex ecosystems, such as freshwater microcosms, will be employed. The final aim would be the treatment of ecosystems where AbR determinants propagate, such as fish farms or hospitals. Despite the promising results observed, identified COINs have some limitations that should be overcome. For example, natural COINs have low chemical stability due to their double bonds susceptible to oxidation (Niki *et al.* 2005). This problem might be solved in controlled environments by combining COINs with antioxidants or a protective atmosphere. In addition, COIN effect was sufficient to prevent plasmid invasion in the absence of selective pressure for plasmid maintenance. The influence of selective pressure should be analyzed, because it is likely that AbR dissemination by vertical gene transfer improves under these conditions. The range of plasmids affected is broad but some plasmids escape from COIN action. IncP and IncI conjugative plasmids are now being employed as test plasmids to screen a collection of bioactive compounds. Therefore, a cocktail of COINs active against different conjugative systems might be applied. As a complement or alternative strategy, some bacteriophages block specific conjugative pilus, with the advantage that they can evolve to counteract a potential emergence of resistant variants.

# Conclusions

1. No genes were found in the chromosome of *E. coli* DH5α that are essential for conjugation of the broad host range plasmid R388. While *E. coli* DH5α presents no barriers against conjugation, most clinically isolated enterobacteria inhibited the entrance of conjugative plasmids, using mechanisms such as bacteriocin production or restriction systems.
2. Two unreported fertility inhibition systems, encoded by IncFI and IncI1 plasmids, substantially reduced conjugation of IncW plasmids.
3. Synthetic 2-alkynoic fatty acids and natural tanzawaic acids were discovered as novel chemical sets of conjugation inhibitors (COINs).
4. Chemical and biological characterization of identified COINs provided the following information:
    a. Compounds with COIN activity contained a carboxylic group and an unsaturated hydrocarbon chain.
    b. IncW and IncF conjugative plasmids, including plasmids mobilized by them, were mainly affected, in most tested host bacteria.
    c. The primary COIN target was found to reside in donor bacteria, probably being part of the mating pair formation system.
    d. The effect of 2-hexadecynoic acid in a bacterial population containing a multi-resistant plasmid demonstrated the feasibility of employing COINs to reduce the prevalence of antibiotic resistance determinants by diminishing horizontal gene transfer in the absence of selective pressure.
    e. Toxicity properties analyzed in bacteria, fungi, and eukaryotic cells positioned natural COINs as potential tools for environmental applications.

# Resumen en español

# 1. Funciones de *Escherichia coli* que limitan la conjugación de plásmidos

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## 1.1. Introducción

La conjugación bacteriana permite la diseminación de diversos rasgos adaptativos entre bacterias alejadas filogenéticamente (Amabile-Cuevas and Chicurel 1992). Entre ellos, destacan los genes de resistencia a antibióticos (Waters 1999, Norman *et al.* 2009), que alcanzan bacterias patógenas ocasionando importantes problemas de salud a nivel mundial (Cooper and Shlaes 2011). Como consecuencia, la búsqueda de nuevas dianas y barreras frente a la conjugación bacteriana podría proporcionar herramientas útiles en la lucha contra la propagación de resistencias.

De forma natural, las defensas bacterianas han evolucionado para hacer frente a genomas invasores potencialmente peligrosos, como bacteriófagos o plásmidos conjugativos. Para ello, usan mecanismos que previenen la entrada de DNA exógeno, como los sistemas de restricción-modificación o CRISPR-Cas (Wilkins 2002, Marraffini and Sontheimer 2008). En esta línea, dos estudios han realizado búsquedas sistemáticas de genes hospedadores implicados en conjugación. En *Escherichia coli*, se utilizó una colección de mutantes como receptores del plásmido R388 (Perez-Mendoza and de la Cruz 2009). Aunque no se encontró ningún gen no esencial imprescindible para la conjugación en sólido, varias mutaciones en genes implicados en la síntesis del lipopolisacárido tuvieron efectos importantes en la conjugación en líquido del plásmido F, lo que realzaba el papel del lipopolisacárido del receptor en la estabilización del par conjugativo. Un trabajo posterior analizó otra colección de mutantes como receptores del ICE*Bs1* de *Bacillus subtilis*, hallando deficiencias conjugativas en mutantes relacionados con la composición de la membrana (Johnson and Grossman 2014).

Con la intención de completar el trabajo anterior (Perez-Mendoza and de la Cruz 2009) y buscar nuevas funciones en el hospedador importantes para la diseminación de plásmidos, se analizó la colección Keio de mutantes de *E. coli* (Baba *et al.* 2006) como donadores de R388, un plásmido de amplio rango de hospedador que puede ser transmitido entre especies filogenéticamente distantes (Mazodier and Davies 1991). Además, se estudió una colección de enterobacterias clínicas como receptoras del mismo plásmido con el fin de estudiar la habilidad de las bacterias patógenas para adquirir nuevos genes de resistencia por conjugación.

## 1.2. Resultados y discusión

**Los mutantes Keio actúan como donadores aptos para la conjugación de R388.** La colección Keio de mutantes de *E. coli* consta de 3.908 mutantes individuales en la mayoría de genes no esenciales (Baba *et al.* 2006). La capacidad donadora de los mutantes fue analizada tras introducir a cada uno de ellos el plásmido pJC01, un derivado de R388 que expresa la proteína GFP únicamente al alcanzar las bacterias receptoras (Getino *et al.* 2015). De esta forma, la frecuencia de conjugación de R388 pudo ser estimada para cada mutante mediante el ratio GFP/$OD_{600}$ tras 6 h de conjugación en medio sólido. Se tomaron al menos dos medidas para cada mutante, que fueron normalizadas frente a la media de la placa de 96 pocillos correspondiente, asumiendo que la mayoría de las mutaciones no afectarían a la capacidad conjugativa del donador. Durante la búsqueda, se sustituyeron 69 mutantes incorrectos detectados por los correspondientes mutantes corregidos (Yamamoto *et al.* 2009). Además, otros 25 mutantes poseían duplicaciones del gen diana, no pudiendo ser delecionado debido a su esencialidad (14 de ellos estaban descritos como esenciales). De hecho, los valores de frecuencias relativas obtenidos se encontraban en el rango entre 0.8 y 1.3, por lo que pudieron considerarse como controles positivos con mayor similitud a los mutantes que a la cepa Keio parental, ya que, a diferencia de ésta, contienen el gen de resistencia a kanamicina que se usó para delecionar el gen correspondiente. Los controles negativos utilizados mostraron valores relativos de 0.03 ± 0.01 en presencia de 2-HDA para reducir la capacidad donadora en el medio (Getino *et al.* 2015) y de 0.74 ± 0.03 si se sobreexpresaba el regulador TrwA en el donador, capaz de disminuir 10 veces la conjugación de R388 (Moncalian *et al.* 1997). Esto indicaría que efectos modestos como el de TrwA en el donador podrían difuminarse durante las 6 h de conjugación necesarias para obtener un máximo de fluorescencia, durante las cuales el mutante sólo actuaría como donador en la primera ronda de conjugación. Sin embargo, un efecto drástico en la capacidad conjugativa podría ser detectable mediante el ensayo utilizado. Como resultado del análisis de la colección Keio, ningún mutante tuvo un efecto drástico sobre la conjugación de R388 (por debajo del 20 % o por encima de 2.4 veces la media). Sólo se encontraron 10 mutantes con la capacidad donadora reducida por debajo de la mitad de la media, y únicamente 5 mostraron frecuencias relativas por encima del doble de la media. Con el propósito de descartar aquellas mutaciones que pudieran ser afectadas por factores de este tipo de ensayo, como la emisión de fluorescencia o los largos tiempos de conjugación, los 20 mutantes más prometedores (10 de ellos con valores relativos por debajo de 0.5 y otros 10 por encima de 1.8) fueron analizados mediante ensayos de conjugación convencional en placa de 1 h y un ratio donador-receptor 1:10. Como control positivo se utilizó el mutante de la DNA polimerasa I (*polA*$^-$), uno de los 14

genes esenciales que no pudieron ser delecionados (Katayama *et al.* 1989), cuyos valores relativos obtenidos en el ensayo primario fueron de 1.1 ± 0.2. Como resultado, varias mutaciones en proteínas de membrana del donador disminuyeron significativamente la conjugación en placa unas 10 veces, de acuerdo con trabajos previos realizados en el receptor (Perez-Mendoza and de la Cruz 2009, Johnson and Grossman 2014). Además, deficiencias en la síntesis de ATP también parecían afectar la conjugación de R388. Como control, la sobreexpresión del regulador TrwA inhibió la conjugación 10 veces, de acuerdo con trabajos anteriores (Moncalian *et al.* 1997). Por tanto, la eficiencia conjugativa parece ligeramente afectada por factores como la composición de la membrana o la disponibilidad de ATP, posiblemente inhibiendo la formación del par conjugativo o la función de las ATPasas implicadas en conjugación.

Como resultado global de ambos estudios, no se encontró ningún gen en el cromosoma del hospedador que fuese esencial para la conjugación de R388. Ni siquiera los mutantes *ihfA*$^{-}$ e *ihfB*$^{-}$ en el factor del hospedador IHF (integration host factor), con funciones estructurales y regulatorias en la conjugación (de la Cruz *et al.* 2010), mostraron valores de conjugación alterados como donador o como receptor, de acuerdo con datos previamente observados (Llosa *et al.* 1991). Si hubiese genes del hospedador necesarios o deletéreos para la conjugación de R388, probablemente fueran esenciales para el crecimiento de *E. coli* o existan genes alternativos con funciones redundantes. Los plásmidos de amplio rango de hospedador pueden resultar útiles para buscar dianas robustas capaces de controlar la conjugación en un amplio rango de especies. Sin embargo, los genes esenciales están evolutivamente más conservados que los no esenciales en bacterias (Jordan *et al.* 2002), por lo que alguno de ellos podría jugar un papel fundamental en la conjugación de estos plásmidos. Es el caso de la DNA polimerasa III, necesaria para la síntesis de la cadena complementaria en donadores y receptores durante la conjugación (Wilkins and Hollom 1974, Kingsman and Willetts 1978). Lo mismo podría ocurrir con otros genes esenciales como *dnaG* (DNA primasa), *polA* (DNA polimerasa I), *rpoD* (factor sigma 70 de la RNA polimerasa), *parC* (DNA topoisomerasa IV) o *rho* (factor de terminación transcripcional) (Katayama *et al.* 1989, Nakamura *et al.* 1983, Joyce and Grindley 1984, Olivera and Bonhoeffer 1974, Das *et al.* 1976), cuyas mutaciones no pudieron ser efectivas (Yamamoto *et al.* 2009) y por tanto sus efectos sobre la conjugación no fueron analizados. Aunque sería interesante definir qué genes esenciales son necesarios para la conjugación, su empleo como diana de la conjugación se traduciría en la búsqueda de nuevos antibióticos, que ejercerían una importante presión selectiva conduciendo rápidamente a la aparición de resistencias.

**Ningún mutante mejora la frecuencia conjugativa de R388.** El trabajo previo se centró en mutaciones del receptor con efectos inhibitorios sobre la conjugación de

R388 (Perez-Mendoza and de la Cruz 2009). Los 40 mutantes en el receptor que mostraron frecuencias de conjugación más altas en ese trabajo fueron reensayados mediante el mismo ensayo, basado en la emisión de luminiscencia en bacterias transconjugantes. Además de los mutantes en el donador previamente seleccionados, se seleccionaron los 10 mutantes en el receptor con frecuencias relativas mayores a 1.5 para descartar posibles efectos del ensayo primario sobre el crecimiento bacteriano, la estabilidad del plásmido o la emisión de luminiscencia. Mediante ensayos de conjugación convencional a un ratio donador-receptor de 1:10, se comprobaron las frecuencias de conjugación de los mutantes seleccionados con respecto al control positivo *polA*$^{-}$. Como resultado, ninguno de los donadores o receptores seleccionados de la colección Keio mostraron mejoras significativas en la frecuencia de conjugación con respecto al control. Esto sugería que las cepas de laboratorio como *E. coli* DH5α no poseían barreras naturales que impidieran la conjugación de plásmidos entre ellas.

**La mayoría de las enterobacterias clínicas bloquean la conjugación de R388.** A diferencia de las cepas de laboratorio, las bacterias adaptadas a ambientes naturales poseen mecanismos de defensa frente a genomas invasores. Utilizando una colección de 316 enterobacterias clínicas como receptores del plásmido R388, se realizó un análisis mediante el ensayo basado en la emisión de luminiscencia en bacterias transconjugantes previamente mencionado. El control positivo con el que se normalizaron los valores obtenidos fue la cepa *E. coli* DH5α vacía como receptor, mientras que como control negativo se sobreexpresó el sistema de exclusión de R388 en dicha cepa (Fernandez-Lopez *et al.* 2005). Estableciendo los valores del control positivo como 100 %, el sistema de exclusión inhibía la entrada de R388 hasta 0.2 %, lo mismo que ocurría en trabajos previos (Perez-Mendoza and de la Cruz 2009). Como resultado del análisis, casi el 80 % de los aislados originales bloquearon la conjugación 10 veces, mientras que sólo un 39 % de los plásmidos transferidos a cepas de laboratorio eran capaces de inhibir la conjugación a ese nivel. Por tanto, la mayor parte de las bacterias analizadas poseían mecanismos en su genoma capaces de controlar la entrada de plásmidos conjugativos.

**Varios plásmidos producen bacteriocinas capaces de matar a los donadores.** Como ejemplos de cepas de laboratorio con plásmidos residentes que inhibían la entrada de R388, se seleccionaron varios procedentes de infecciones del tracto urinario (Ejrnaes *et al.* 2011) que habían sido transferidos a *E. coli* DH5α (Alvarado 2016). No se detectaron relaxasas del grupo $MOB_{F11}$ en ninguna de las cepas originales (Alvarado *et al.* 2012, Alvarado 2016), por lo que una inhibición por incompatibilidad replicativa o sistemas de exclusión era improbable. Sin embargo, la mayoría de los receptores seleccionados poseían plásmidos de los grupos $MOB_{P51}$ y $MOB_{C11}$, típicamente productores de colicinas. De hecho, al realizar un test de inhibición del crecimiento de la

cepa *E. coli* DH5α, se observaron halos inhibitorios alrededor de los cultivos filtrados de todos aquellos receptores que contenían plásmidos de esos grupos. De esa forma, las bacterias receptoras mataban a las donadoras, que eran susceptibles a esos agentes antimicrobianos, inhibiendo indirectamente la conjugación de R388.

**Ciertas *E. coli* patógenas inhiben la transferencia génica horizontal mediante sistemas de restricción-modificación.** Como ejemplo de cepa original capaz de inhibir la conjugación de R388, se seleccionó una *E. coli* patógena aislada de infecciones urinarias (Ejrnaes *et al.* 2011). Según trabajos anteriores, la cepa 3065 seleccionada poseía un plásmido resistente a ampicilina del grupo $MOB_{P12}$ (Alvarado *et al.* 2012, Alvarado 2016). Cuando ese plásmido fue transferido a una cepa de laboratorio, la inhibición desaparecía, por lo que el mecanismo no estaba presente en dicho plásmido. La influencia del ensayo primario fue descartada mediante conjugación en placa, utilizando cuatro plásmidos conjugativos de los grupos IncW, IncFI, IncFII e IncP1. A diferencia de lo que ocurría usando *E. coli* DH5α como recipiente, la conjugación de los cuatro plásmidos disminuía más de 4 órdenes de magnitud si el recipiente era *E. coli* 3065. Para comprobar si este mecanismo de inhibición de amplio espectro también se extendía a otros tipos de transferencia horizontal, se transformó esa misma cepa (y *E. coli* DH5α como control) con tres plásmidos de diferentes tamaños. Cuando los plásmidos se extrajeron de la cepa control, sólo resultó inhibida la transformación de 3065 con los dos plásmidos más grandes. Además, si esos dos plásmidos eran obtenidos de la cepa 3065, las frecuencias de transformación se restauraban a niveles del control. Estos resultados indicaban la presencia de un sistema de restricción-modificación como mecanismo inhibidor de la conjugación y la transformación. Por un lado, los plásmidos más afectados, por su tamaño, tendrían más posibilidades de contener dianas de restricción que afectaran la transformación de 3065. Y por otro lado, la cepa que potencialmente contenía el sistema de restricción, podría modificar esas dianas como lo haría para la protección de su genoma, de forma que la transformación con plásmidos extraídos de ella se restauraría, explicando de esta forma los resultados obtenidos. Según un estudio reciente (Oliveira *et al.* 2014), el 69 % de los genomas procarióticos contienen sistemas de restricción como sistemas de defensa. Además, muchos plásmidos conjugativos poseen proteínas anti-restricción para hacer frente a esos sistemas, como ArdC de R388 (Belogurov *et al.* 2000). Todos estos datos sugieren que los sistemas de restricción son importantes armas en la lucha contra los genomas invasores.

# 2. Inhibición de la fertilidad entre plásmidos conjugativos

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## 2.1. Introducción

Las bacterias poseen mecanismos para controlar la conjugación bacteriana que podrían ser empleados para prevenir la diseminación de resistencias a antibióticos (Norman *et al.* 2009). Entre ellos, los sistemas de restricción-modificación y CRISPR-Cas actúan como sistemas inmunes bacterianos frente a genomas invasores, como los bacteriófagos o los plásmidos conjugativos (Wilkins 2002, Marraffini and Sontheimer 2008). Los propios plásmidos conjugativos también pueden presentar funciones capaces de bloquear la conjugación. Los sistemas de exclusión impiden la entrada de plásmidos similares en una misma bacteria, con el fin de evitar la competición entre ellos y excesivas rondas de conjugación que puedan producir demasiado gasto energético o dañar la membrana del receptor (Garcillan-Barcia and de la Cruz 2008). Algunos plásmidos inhiben su propia conjugación con el fin de minimizar la carga asociada a la expresión constitutiva del sistema conjugativo, que incluye la vulnerabilidad a ciertos bacteriófagos. Es el caso del sistema FinOP de los plásmidos IncF, que también puede actuar sobre otros plásmidos con sistemas de regulación similares (Frost and Koraimann 2010).

Ciertos plásmidos codifican sistemas de inhibición de la fertilidad capaces de reducir la transferencia de otros plásmidos no relacionados presentes en el mismo donador. Se ha sugerido un posible papel relacionado con una mejor competitividad para colonizar nuevos hospedadores (Gasson and Willetts 1975, 1977). Por ejemplo, FinC del plásmido CloDF13, que posee su propia proteína acopladora, podría estar inhibiendo la función de la proteína acopladora del plásmido que lo moviliza en favor de su propia movilización (Willetts 1980). En total, se conocen diez genes responsables de inhibir la fertilidad de plásmidos del grupo IncF (*finQ*, *finW*, *finC*, *finU* y *finV*) (Gasson and Willetts 1975), IncW (*fiwA* y *fiwB*) (Fong and Stanisich 1989), IncP (*fipA* y *pifC*) (Winans and Walker 1985, Miller *et al.* 1985) y del plásmido pTi de *Agrobacterium tumefaciens* (*osa*) (Close and Kado 1991). Aunque inicialmente se estableció la proteína acopladora de los plásmidos IncP como diana de FipA y PifC (presente en plásmidos IncN e IncF respectivamente) (Santini and Stanisich 1998), recientemente se ha observado una actividad endonucleasa en la proteína Osa del plásmido R388, capaz de degradar el T-DNA del plásmido pTi (Maindola *et al.* 2014).

Con el fin de encontrar nuevos mecanismos de inhibición de la fertilidad que puedan servir en la lucha frente a la diseminación de resistencias a antibióticos, o revelar información útil para el uso de la conjugación como herramienta biotecnológica, el trabajo se ha centrado en el estudio de interacciones entre sistemas conjugativos del grupo IncW y un conjunto representativo de plásmidos co-residentes no relacionados.

## 2.2. Resultados y discusión

**Cuatro grupos de plásmidos co-residentes inhiben la transferencia de R388.** El grupo de plásmidos de amplio rango de hospedador IncW se ha estudiado en presencia de un conjunto de plásmidos conjugativos no relacionados presentes en el mismo donador. Se han analizado tanto variantes naturales de plásmidos representativos del grupo, como variantes sintéticas reducidas de R388 que contenían los genes indispensables para la conjugación. Las frecuencias de conjugación para cada plásmido fueron normalizadas frente a la media obtenida en ausencia de los plásmidos co-residentes. Como variantes naturales se utilizaron los plásmidos R388 o pIE321, dependiendo de las resistencias del plásmido co-residente. La conjugación de R388 resultó significativamente inhibida en presencia de cuatro grupos de incompatibilidad. En concreto, la transferencia se redujo unos 4-log en presencia de la versión reducida de F pOX38 (IncFI), de R64 (IncI1), del derivado de RP4 pRL443 (IncP1α), y del plásmido IncX2 R6K. Aunque algunas interferencias observadas ya habían sido descritas anteriormente, como es el caso de la inhibición por plásmidos IncP1α debida a los genes *fiwA* y *fiwB* (Fong and Stanisich 1989) o el mecanismo no identificado presente en R6K (Olsen and Shipley 1975), las interferencias causadas por los plásmidos IncFI e IncI1 eran nuevas. El resto de plásmidos co-residentes analizados (pertenecientes a los grupos IncFII, IncP1β, IncX1, IncL/M e IncH) no produjeron cambios significativos en la conjugación de R388 o pIE321, sólo pKM101 (IncN) mejoró 3 veces la conjugación de R388. En cuanto al efecto de los plásmidos IncW estudiados en la conjugación de los plásmidos co-residentes, solamente los plásmidos IncP1 pRL443 y R751 resultaron afectados entre 10 y 100 veces, un efecto similar al observado en el plásmido IncP1 RP1 en presencia de R388 (Olsen and Shipley 1975).

**La versión sintética de R388 es más resistente al efecto de pOX38 que la variante natural.** Además de las variantes naturales del grupo IncW, se analizó el comportamiento de una versión sintética reducida de R388 en presencia de los mismos plásmidos conjugativos. La intención de este experimento era superar los obstáculos observados en presencia de esos cuatro grupos de plásmidos y poder usar R388 como "cable" en computación bacteriana para transferir información en combinación con otros plásmidos conjugativos (Goni-Moreno *et al.* 2013, Amos *et al.* 2015). Para ello, se utilizó

la cepa de genoma reducido MDS42 (Posfai *et al.* 2006), en la que se habían insertado los genes encargados de la formación del par conjugativo (*MPF*) (Garcillán-Barcia, no publicado). Mediante este MPF sintético, tres plásmidos movilizables que contenían el origen de transferencia y los genes encargados el procesamiento del DNA (*MOB*) de R388 fueron analizados en presencia de los distintos plásmidos co-residentes, usando uno u otro dependiendo de las resistencias que expresaran éstos. Aunque los efectos inhibitorios observados utilizando este sistema sintético fueron similares a los descritos con el sistema natural, los efectos producidos por pOX38 y R64 fueron dos órdenes de magnitud más leves, diferencias que podría aportar claves importantes sobre el mecanismo de inhibición hallado. Además, la movilización del IncW sintético resultó inhibida 10 veces en presencia de los plásmidos R1drd19 y pCTX-M3, y el IncW sintético afectó a la conjugación de todos los plásmidos co-residentes, mejorándola en unos casos (R751 y pOLA52) y empeorándola en otros (pKM101, pOX38, R1drd19, R100-1, R64, pRL443, R6K, pCTX-M3 y drR27).

**La sobreexpresión de la proteína acopladora de R388 alivia parcialmente la inhibición producida por pOX38.** La restauración parcial del efecto inhibitorio de pOX38 sobre R388 cuando se usaba la versión sintética podría deberse al aumento en el número de copias de los genes *MOB*. Además, el número de copias del *MPF* sintético era 1 por estar insertado en el cromosoma, frente a las 2-3 de la variante natural de R388, por lo que el número de copias relativo de los genes *MOB* se veía más incrementado aún. Considerando estos resultados y el hecho de que se había descrito previamente que los factores de inhibición de la fertilidad FipA, PifC y Osa tenían como diana la proteína acopladora (Santini and Stanisich 1998, Cascales *et al.* 2005), se decidió testar el efecto de la sobreexpresión de TrwB (la proteína acopladora de R388) en presencia de la combinación de plásmidos R388 y pOX38. Sin embargo, la conjugación de R388 sólo mejoró 6 veces con respecto al control en ausencia de TrwB, por lo que la estequiometría de los otros genes *MOB trwA* y *trwC* e incluso del *oriT* también parecen importantes para la mejora en la transferencia del R388 sintético. Conociendo el último trabajo que apunta a un modo de acción de Osa relacionado con la degradación del T-DNA (Maindola *et al.* 2014), el efecto observado podría deberse simplemente al aumento en la cantidad de DNA plasmídico que puede ser transferido. Ese mismo artículo apunta a un mecanismo común de inhibición de la fertilidad, ya que distintas proteínas inhibidoras de la fertilidad, tanto homólogas a Osa (ICE1056Fin y FiwA) como no relacionadas filogenéticamente (FipA y PifC), inhiben la transferencia del plásmido pTi. La presencia del gen *pifC* en F y su versión reducida pOX38, que inhibe la fertilidad de plásmidos IncP (Miller *et al.* 1985), podría indicar su implicación en la inhibición de R388. La presencia de un homólogo 99 % idéntico en R64 apoya esta hipótesis, ya que también es capaz de inhibir la conjugación de R388 y RP4 (Datta *et al.* 1971). Además,

la inhibición causada por pOX38 y R64 se ve aliviada en ambos casos al sustituir R388 por su variante sintética, lo que también podría sugerir un mecanismo de acción común. De la misma forma, la ausencia de *pifC* en los plásmidos IncFII, sin efectos sobre la conjugación de R388, está de acuerdo con esta afirmación. Sin embargo, la presencia de los genes *fipA* o *pifC* de pKM101 y F respectivamente, suficientes para inhibir la conjugación de RP4, no afectaron la conjugación de R388 en un estudio previo (Santini and Stanisich 1998), lo que descartaría *pifC* como el gen responsable de la inhibición de R388 por pOX38 y R64.

**El plásmido sintético pX1.0 es más sensible a la inhibición de la fertilidad que la variante natural pOLA52.** La versión sintética de pOLA52, denominada pX1.0, fue construida para representar el esqueleto común de los plásmidos IncX1 (Hansen *et al.* 2011). Del mismo modo que se analizaron las diferencias entre la variante natural y sintética de R388, se estudiaron las dos variantes IncX1 en presencia de los cuatro plásmidos con resistencias compatibles: R388 (IncW), pOX38 (IncFI), R64 (IncI1) y R751 (IncP1β). Como resultado, los plásmidos R751 y R64 inhibieron la conjugación de pX1.0 100 y 10 veces respectivamente, mientras que pOLA52 no resultó afectado. Además, pX1.0 mejoró 4 veces la conjugación de su co-residente R388.

El estudio de la red de interacciones entre plásmidos puede allanar el camino en el futuro desarrollo de herramientas biotecnológicas que utilicen la conjugación como sistema de transferencia de información en bacterias. Además, un mecanismo de inhibición conservado entre sistemas conjugativos podría ser explotado en la lucha contra la diseminación de resistencias a antibióticos.

# 3. Ácidos grasos sintéticos que inhiben la transferencia génica horizontal mediada por plásmidos

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## 3.1. Introducción

La conjugación bacteriana es el principal mecanismo de transferencia génica horizontal responsable de la diseminación de genes de resistencia a antibióticos (Pitout 2010). Estos genes de resistencia son capaces de alcanzar rápidamente patógenos humanos, limitando la eficacia de cada vez más antibióticos frente a infecciones bacterianas, lo que ha ocasionado un grave problema a nivel mundial (WHO 2014). Con el fin de evitar la propagación de resistencias, se han propuesto varias estrategias centradas en controlar la conjugación de plásmidos portadores (Smith and Romesberg 2007, Baquero *et al.* 2011).

Algunos ejemplos incluyen el uso de inhibidores específicos de componentes clave de la conjugación, como pueden ser la relaxasa o el pilus conjugativo. La relaxasa es la enzima iniciadora de la conjugación, responsable de realizar un corte en una cadena del origen de transferencia del plásmido que va a ser movilizado a la bacteria receptora (de la Cruz *et al.* 2010). La producción de anticuerpos específicos frente a la relaxasa del plásmido R388 bloqueó su actividad en la bacteria receptora y en consecuencia, la conjugación de este plásmido, confirmando también su papel en el receptor (Garcillan-Barcia *et al.* 2007). Otro trabajo utilizó bisfosfonatos como inhibidores específicos del centro activo de la relaxasa del plásmido F (Lujan *et al.* 2007). Sin embargo, su acción sobre la conjugación fue posteriormente atribuida a su actividad quelante, lo que tenía un efecto negativo en el crecimiento bacteriano (Nash *et al.* 2012). El pilus conjugativo es un componente esencial para la transferencia del plásmido, ya que pone en contacto las bacterias donadora y receptora (Clarke *et al.* 2008). Algunos virus que infectan bacterias, denominados bacteriófagos, tienen como diana primaria el pilus expresado por ciertos plásmidos conjugativos. Explotando esta afinidad natural, el bacteriófago M13 bloqueó específicamente el pilus del plásmido F, inhibiendo el contacto donador-receptor y por tanto, su conjugación. Lo mismo ocurría al utilizar la proteína pIII encargada del reconocimiento del pilus (Lin *et al.* 2011).

Como estrategia alternativa, se ha hecho una búsqueda sistemática de compuestos inhibidores de la conjugación (COINs) utilizando un ensayo automatizado para estimar la frecuencia de conjugación mediante la detección de luminiscencia en las células

receptoras que han adquirido el plásmido. Realizando este ensayo en presencia de la colección NatChem de 12.000 compuestos naturales, se descubrió que ciertos ácidos grasos insaturados, como el ácido oleico y linoleico, eran capaces de inhibir la conjugación de los plásmidos R388 y pOX38 (pertenecientes a los grupos de incompatibilidad IncW e IncFI respectivamente), sin reducir el crecimiento bacteriano (Fernandez-Lopez *et al.* 2005). El COIN más efectivo fue el ácido dehidrocrepenínico, pero su extracción a partir de plantas tropicales (Gussoni *et al.* 1994) complicaba su caracterización.

En este trabajo se han sintetizado ácidos grasos de estructura simple capaces de inhibir la conjugación de varios grupos de plásmidos en diferentes especies bacterianas. En concreto, el ácido 2-hexadecinoico (2-HDA) bloqueó la propagación del plásmido multi-resistente R1drd19 en una población bacteriana, demostrando así su potencial para controlar la diseminación de resistencias a antibióticos.

## 3.2. Resultados y discusión

**El 2-HDA es el COIN sintético más efectivo**. Se sintetizaron una serie de ácidos grasos 2-alquinoicos (2-AFAs) de estructura simple (Tasdemir *et al.* 2010, Carballeira *et al.* 2012, Sanabria-Rios *et al.* 2014) con el fin de estudiar las propiedades químicas necesarias para obtener una actividad inhibidora de la conjugación. Para ello se utilizó un plásmido fluorescente derivado de R388 que únicamente producía la proteína GFP en las bacterias transconjugantes, donde la polimerasa del fago T7 reconocía su promotor. De esta forma, en presencia de un COIN, la fluorescencia detectada era menor. En primer lugar se analizó la longitud de la cadena de carbonos sin variar el triple enlace en el segundo carbono, observándose este orden en la potencia de mayor a menor: 2-HDA (16 C) > ácido 2-octadecinoico (2-ODA, 18 C) > ácido 2-tetradecinoico (14 C) > ácido 2-icosinoico (20 C) > ácido 2-dodecinoico (12 C). El más efectivo fue el 2-HDA, capaz de reducir hasta un 2 % la conjugación del plásmido R388 a una concentración de 0.3 mM. Por otro lado, se sintetizaron otra serie de análogos, partiendo tanto del 2-HDA como del 2-ODA y sustituyendo su grupo carboxílico por otros grupos funcionales con el fin de comprobar cuáles eran importantes para la actividad observada. Analizando estos análogos, entre los que se incluían 2-alquinoles, metil 2-alquinoatos y tetrahidropiranil-éteres, se concluyó que sólo eran activos aquellos que tenían el grupo carboxilo intacto. Además, se sintetizaron dos derivados diinsaturados del 2-HDA, el 2,6-HDA y el 2,9-HDA. El examen de estos dos compuestos reveló la importancia de la posición de un segundo triple enlace, ya que el primero seguía siendo activo (aunque era menos potente que el 2-HDA), mientras que el que presentaba una segunda insaturación más alejada del grupo carboxilo resultó inactivo. Por lo tanto, la presencia de un grupo

carboxílico y una cadena insaturada de determinada longitud, así como la posición de las insaturaciones son características clave para que un compuesto posea actividad COIN.

**Los COINs sintéticos inhiben la transferencia de varios grupos de plásmidos.** Además de R388, otros plásmidos fueron afectados por los COINs identificados. La conjugación de todos los plásmidos pertenecientes a los grupos IncW, IncF e IncH analizados mediante ensayos de conjugación convencionales en medio sólido resultó inhibida aproximadamente 100 veces en presencia de 2-HDA 0.4 mM. Aunque los plásmidos IncN e IncP fueron totalmente resistentes al compuesto, algunos grupos mostraron cierta sensibilidad al utilizar concentraciones más elevadas de 2-HDA (IncI, IncL/M e IncX). Además de revelar el alcance de la actividad, el rango particular de plásmidos afectados podría aportar indicios sobre el mecanismo de inhibición, probablemente compartido con los COINs anteriormente descritos, con los que también comparten características estructurales, de potencia y rango de actividad (Fernandez-Lopez *et al.* 2005). Los plásmidos movilizables también pueden ser portadores de resistencias, aunque necesitan la maquinaria de transporte de un plásmido conjugativo para ser movilizados (Francia *et al.* 2004). Utilizando varios plásmidos movilizables en combinación con diferentes plásmidos conjugativos, se observaron efectos inhibitorios en la movilización de aquellos plásmidos que eran transferidos gracias a la ayuda de plásmidos conjugativos previamente descritos como sensibles a los COINs. Sin embargo, cuando el mismo plásmido movilizable era transferido gracias a la maquinaria de un plásmido conjugativo resistente al inhibidor, el plásmido movilizable se transfería con normalidad. Estos resultados sugieren una diana común perteneciente al sistema de formación del par conjugativo (MPF) que poseen únicamente los plásmidos conjugativos para formar el canal que conecta donador y receptor. Asimismo, la movilización de la región *oriT-MOB* de R388 (Fernandez-Gonzalez *et al.* 2011) mediante el sistema MPF del plásmido pKM101 (Draper *et al.* 2005), resistente al efecto COIN, reafirmó esta hipótesis al no mostrar efectos inhibitorios en presencia de 2-HDA. En esta línea, cabe mencionar la inhibición observada en presencia de ácido linoleico de la actividad ATPasa de TrwD, una proteína involucrada en la síntesis del MPF de R388 (Machon 2004).

**Los COINs actúan sobre distintas especies de bacterias donadoras.** Preincubando las células donadoras y receptoras por separado con 2-HDA y realizando una conjugación convencional en ausencia del inhibidor, se observó inhibición únicamente cuando los donadores fueron preincubados, mientras que la preincubación de los receptores no tuvo ningún efecto sobre la conjugación. Este resultado situó a los donadores como dianas primarias de la actividad COIN. Con la intención de averiguar si el efecto se extendía a otras especies bacterianas además de *Escherichia coli*, se introdujo el plásmido de amplio rango de hospedador R388 en *Salmonella enterica,*

*Acinetobacter baumannii*, *Vibrio cholerae*, *Agrobacterium tumefaciens* y *Pseudomonas putida*. Utilizando estas especies como donadoras en ensayos de conjugación convencionales, la conjugación de R388 se inhibió en todos los casos en presencia de 2-HDA. En el caso de *V. cholerae*, su crecimiento también se vio afectado, por lo que el efecto observado no pudo ser atribuido específicamente a la actividad COIN. La conjugación del plásmido IncFII pSLT también se vio afectada en presencia de 2-HDA utilizando tanto *E. coli* como *S. enterica* como donador o receptor. Por tanto, la actividad COIN parece independiente del hospedador utilizado, extendiendo así el rango de bacterias sobre el que podría ser empleado.

**La capacidad invasora del plásmido R1drd19 es suprimida en presencia de 2-HDA.** La utilidad de los COINs en la lucha contra la diseminación de resistencias a antibióticos fue analizada mediante conjugación en medio líquido del plásmido desreprimido R1drd19, representante del grupo portador de resistencias más importante en enterobacterias patógenas, el grupo de incompatibilidad IncF (Carattoli 2009). La población bacteriana inicial contenía un ratio 1:1 donador-receptor, proporción que fue testada mediante réplica en placa a lo largo de varias generaciones en presencia de diferentes concentraciones de 2-HDA. En ausencia de 2-HDA, el plásmido invadía la población en cuestión de cuatro generaciones, ya que la carga genética que ejercía sobre el donador disminuyendo su velocidad de crecimiento era compensada por su alta infectividad, transfiriéndose rápidamente a las bacterias receptoras. Sin embargo, la adición de un inhibidor de la conjugación como el 2-HDA provocó la inversión del equilibrio carga-infectividad (Stewart and Levin 1977). De esta forma, en presencia de 2-HDA 0.4 mM, el plásmido no sólo era incapaz de invadir la población bacteriana, sino que la carga que ejercía sobre el hospedador superaba su capacidad infectiva inhibida de tal forma que la proporción inicial de células con plásmido (50 %) decaía al 27 % a lo largo de 15 generaciones. Para interpretar estos resultados, se construyó un modelo simple de la prevalencia del plásmido en la población determinada por las ganancias vía conjugación y el efecto competitivo entre bacterias con y sin plásmido en ausencia de presión selectiva. Ajustando los resultados experimentales al modelo teórico se pudo extraer una tasa de conjugación aparente para cada concentración de 2-HDA, lo que mostró una $IC_{50}$ de aproximadamente 50 μM, equivalente a la observada en los ensayos de conjugación del plásmido R388. Por tanto, el 2-HDA no es únicamente capaz de bloquear la capacidad invasiva de un plásmido conjugativo, sino también de reducir su prevalencia en poblaciones bacterianas a concentraciones que inhiben la conjugación tan sólo al 50 % en ausencia de presión selectiva.

# 4. Ácidos tanzawaicos, un nuevo grupo químico de inhibidores de la conjugación bacteriana

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## 4.1. Introducción

Las infecciones causadas por patógenos resistentes a antibióticos provocan numerosas víctimas anualmente en todo el mundo (Hawkey and Jones 2009). La diseminación de resistencias en enterobacterias ocurre mayoritariamente mediante conjugación bacteriana (Halary *et al.* 2010, Norman *et al.* 2009), por lo que podría ser controlada por moléculas que interfieren con este proceso (Baquero *et al.* 2011, Fernandez-Lopez *et al.* 2005).

Se ha intentado controlar la conjugación bloqueando componentes específicos de la conjugación (Lujan *et al.* 2007, Garcillan-Barcia *et al.* 2007, Lin *et al.* 2011) o el proceso conjugativo global (Getino *et al.* 2015, Fernandez-Lopez *et al.* 2005). Entre los compuestos inhibidores (COINs) descubiertos hasta la fecha se encuentran el ácido dehidrocrepenínico (Fernandez-Lopez *et al.* 2005), que debe ser extraído de semillas de plantas tropicales (Gussoni *et al.* 1994), los ácidos insaturados con dobles enlaces susceptibles a la oxidación (Niki *et al.* 2005), como son el ácido oleico y linoleico (Fernandez-Lopez *et al.* 2005), y los ácidos grasos sintéticos 2-hexadecinoico (2-HDA) y 2-octadecinoico (2-ODA) (Getino *et al.* 2015), fácilmente sintetizables (Tasdemir *et al.* 2010, Carballeira *et al.* 2012, Sanabria-Rios *et al.* 2014) pero con problemas de toxicidad en hongos (Gershon and Shanks 1978, Carballeira *et al.* 2006), protozoos (Tasdemir *et al.* 2010, Carballeira *et al.* 2012), algunas bacterias y células eucariotas (Konthikamee *et al.* 1982, Morbidoni *et al.* 2006, Sanabria-Rios *et al.* 2014).

Con el fin de encontrar nuevos COINs con mejores propiedades para su aplicación práctica, se realizó una búsqueda sistemática utilizando la colección AQUAc de compuestos bioactivos aislados de microorganismos marinos. Como resultado, se descubrieron dos nuevos COINs naturales, los ácidos tanzawaicos (TZAs) A y B, con toxicidad reducida respecto a los sintéticos y capaces de inhibir la conjugación de una importante fracción de plásmidos.

## 4.2. Resultados y discusión

**Los TZAs A y B inhiben la conjugación de R388.** La colección AQUAc de compuestos parcialmente purificados, extraídos principalmente de actinomicetos, hongos y micro-algas de origen marino, fue analizada utilizando un sistema de búsqueda basado en la emisión de luminiscencia del plásmido R388 en bacterias transconjugantes, donde el promotor *lac* del operón *lux* es liberado del represor LacI (Fernandez-Lopez *et al.* 2005). De los 1.632 compuestos analizados, 9 fueron seleccionados para un ensayo control que descartaba aquellos que afectaran al crecimiento, la estabilidad del plásmido o la emisión de luminiscencia, utilizando bacterias que contenían un plásmido que expresaba constitutivamente el operón *lux*. Dos compuestos prometedores, P515 y P605, fueron seleccionados tras llevar a cabo ensayos de potencia y confirmación de la actividad por conjugación convencional. Las cepas productoras fueron re-fermentadas con el fin de purificar los compuestos activos mediante un fraccionamiento guiado por un ensayo de conjugación basado en la emisión de fluorescencia, capaz de descartar directamente fracciones que afectaran al crecimiento bacteriano (Getino *et al.* 2015). Mientras que la cepa productora de P515 no permitió la purificación de ningún compuesto activo, el fraccionamiento de P605 dio lugar a un compuesto activo, cuya estructura fue resuelta por resonancia magnética nuclear. El nuevo COIN fue identificado como TZA-B, un poliquétido previamente descrito como inhibidor de la producción de aniones superóxido extraído a partir de *Penicillium citrinum* (Kuramoto *et al.* 1997, Malmstrom *et al.* 2000). El análisis dosis-respuesta determinó que el TZA-B reducía la conjugación de R388 hasta un 2 % a una concentración de 0.4 mM, datos similares a los observados en presencia de los COINs previamente descritos (Fernandez-Lopez *et al.* 2005, Getino *et al.* 2015). Dos análogos estructurales inhibidores de la producción de aniones superóxido (Kuramoto *et al.* 1997, Malmstrom *et al.* 2000), TZA-A and TZA-E, fueron también estudiados. El TZA-A tuvo efectos similares al TZA-B sobre la conjugación de R388. Sin embargo, el TZA-E, que poseía un grupo hidroxilo adicional, no mostró actividad COIN. Por tanto, aunque químicamente más complejos que los ácidos grasos insaturados (poseen dos anillos aromáticos), los TZAs comparten ciertas características con ellos, como el grupo carboxílico y la cadena insaturada, confirmando la importancia de estas dos características para observar este tipo de actividad. Además, la adición de un único grupo hidroxilo en el extremo opuesto al grupo carboxílico es capaz de anular la actividad inhibitoria que parece producir la combinación de estas dos características químicas.

**Las principales dianas de los TZAs son los plásmidos IncW e IncF.** El rango de plásmidos susceptibles fue estudiado mediante el análisis de una colección de plásmidos conjugativos clínicamente representativos de enterobacterias. Los plásmidos más

afectados por la adición de TZA-B 0.4 mM fueron R388 (IncW) y R100-1 (IncFII), que mostraron reducciones en su conjugación en torno a 100 veces. Aunque en menor medida, pOX38 (IncFI), R1drd19 (IncFII), R64drd11 (IncI), pCTX-M3 (IncL/M), R6K (IncX) y drR27 (IncH) también resultaron inhibidos hasta valores de entre un 10 y un 50 % la frecuencia de conjugación en ausencia del compuesto. Como ocurría con los COINs previamente descritos (Fernandez-Lopez *et al.* 2005, Getino *et al.* 2015), el TZA-B no logró reducir la conjugación de los plásmidos pertenecientes a los grupos IncN (pKM101) e IncP (pRL443 y R751). Del mismo modo, la transferencia de los plásmidos movilizables analizados sólo resultó inhibida cuando el plásmido conjugativo utilizado para aportar el sistema formador del par conjugativo (MPF) era sensible a la acción del TZA-B. Como ejemplo, la movilización del plásmido ColE1 era susceptible al efecto del COIN cuando se utilizaron los plásmidos R388 (IncW) y R100-1 (IncFII), mientras que su movilización por pRL443 (IncP) resultó resistente al efecto del inhibidor, lo mismo que ocurría con el propio pRL443. Estos resultados sugieren que la diana del TZA-B también se encontraría en el sistema MPF que comparten los plásmidos conjugativos y movilizables para su transferencia.

**Los COINs naturales poseen una toxicidad reducida.** La toxicidad de los COINs descritos hasta el momento fue analizada en diferentes organismos con el fin de seleccionar aquellos que afectaran mínimamente la biodiversidad del ecosistema a tratar. Mientras que los COINs sintéticos 2-HDA y 2-ODA mostraron actividad bactericida en bacterias Gram positivas y hongos a bajas concentraciones (~10 μM), ninguno de los COINs naturales (TZA-A, TZA-B, ácidos oleico y linoleico) mostró efectos tóxicos ($IC_{90}$) en las bacterias y hongos testados por debajo de 100 μM. En cambio, la citotoxicidad ($IC_{50}$) observada frente a las cinco líneas celulares humanas analizadas se encontraba en torno a 100 μM en todos los COINs descritos. Teniendo en cuenta la eficacia demostrada de los COINs para prevenir la diseminación de plásmidos conjugativos en poblaciones bacterianas a concentraciones de inhibidor de hasta 50 μM (Getino *et al.* 2015), los valores de toxicidad observados en los COINs naturales podrían considerarse permisibles para el tratamiento de ambientes complejos. Aunque los COINs naturales son potencialmente menos estables (Niki *et al.* 2005), algunos de ellos ya forman parte de la dieta humana (Di Vaio *et al.* 2013), por lo que su aplicación práctica parece más factible.

# Conclusiones en español

1. No se encontraron genes en el cromosoma de *E. coli* DH5α esenciales para la conjugación del plásmido de amplio rango de hospedador R388. Mientras que *E. coli* DH5α no presentaba barreras contra la conjugación, la mayoría de las enterobacterias clínicas inhibieron la entrada de plásmidos conjugativos, usando mecanismos como la producción de bacteriocinas o los sistemas de restricción.
2. Dos nuevos sistemas de inhibición de la fertilidad, codificados en plásmidos IncFI e IncI1, disminuyeron sustancialmente la conjugación de los plásmidos IncW.
3. Se descubrieron dos nuevos grupos químicos de inhibidores de la conjugación (COINs), los ácidos 2-alquinoicos sintéticos y los ácidos tanzawaicos naturales.
4. La caracterización química y biológica de los COINs identificados proporcionó la siguiente información:
    a. Los compuestos con actividad COIN contenían un grupo carboxílico y una cadena hidrocarbonada insaturada.
    b. Los plásmidos conjugativos IncW e IncF, incluyendo los plásmidos movilizados por ellos, fueron principalmente afectados, en la mayor parte de bacterias hospedadoras analizadas.
    c. La diana COIN primaria se encontraba en la bacteria donadora, probablemente en el sistema de formación del par conjugativo.
    d. El efecto del ácido 2-hexadecinoico en una población bacteriana que contenía un plásmido multi-resistente demostró la posibilidad de emplear COINs para reducir la prevalencia de determinantes de resistencia a antibióticos, disminuyendo la transferencia génica horizontal en ausencia de presión selectiva.
    e. Las propiedades tóxicas analizadas en bacterias, hongos y células eucariotas situó a los COINs naturales como herramientas potenciales para aplicaciones medioambientales.

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