A search for new mechanisms to inhibit plasmid conjugation

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Abstract

BACKGROUND: Infections due to antibiotic-resistant (AbR) bacteria are a major cause of morbidity and mortality throughout the world. In addition, the number of new antibiotics being developed has plummeted. Although resistance genes can disseminate by any horizontal gene transfer mechanism, the vast majority of reports of bacterial gene transfer in the environment involve conjugation. Our group developed a method for high-throughput analysis of conjugation. This method was used to check for host genes in the recipient cell involved in conjugation, concluding that lab strains of *Escherichia coli* like DH5α have no non-essential genes that play an essential role in conjugation. In recipient cells, conjugation can be inhibited by different mechanisms: restriction systems, CRISPRs, entry exclusion systems or incompatibility, among others. Our aim is to screen a set of natural plasmids to look for conjugation broad-range inhibitor genes in enterobacterial plasmids, in order to hopefully identify new mechanisms of inhibition that could be used to control AbR propagation.

RESULTS: Most clinical isolates analyzed have mechanisms that inhibit plasmid conjugation at least indirectly. In a minority, these mechanisms are encoded by plasmids that can be transferred to a lab *E. coli* strain. Some of these plasmids encode genes for the synthesis of colicins, which inhibit the growth of the donor strain. We found at least one plasmid that inhibited, not the cell growth, but the conjugation of several plasmids.

FUTURE RESEARCH: Once a plasmid inhibiting conjugation was found, the next step will be to identify the gene or genes responsible for this inhibition. We will try direct cloning of the responsible genes as well as random mutagenesis by transposition.

Introduction

Horizontal gene transfer

DNA composition of organisms can be extraordinary variable, due to DNA fragments can be transferred from one organism to another, and can be incorporated stably in the receptor, permanently changing its genetic composition. This process is called horizontal gene transfer (HGT; Bushman, 2002). Since Barbara McClintock in the 40s, after discovering the existence of transposable elements in maize, propose that genomes are dynamic, it has proved the flow of genes across multiple organisms in the laboratory. In addition, by comparing genome sequences, it has been proposed that gene transfer has occurred among distant organisms or even unrelated ones in evolution: among bacteria (Jain et al., 1999), from bacteria to eukaryotes (Doolittle, 1998), from bacteria to archaea (Nelson et al., 1999) and from animals to bacteria (to intracellular bacterial parasites) (Wolf et al., 1999). But without doubt, is among bacteria where HGT occurs more frequently. This exchange of information outweighs the prokaryotic clone mode and affects bacterial adaptation, speciation and evolution (Gogarten y Townsend, 2005).

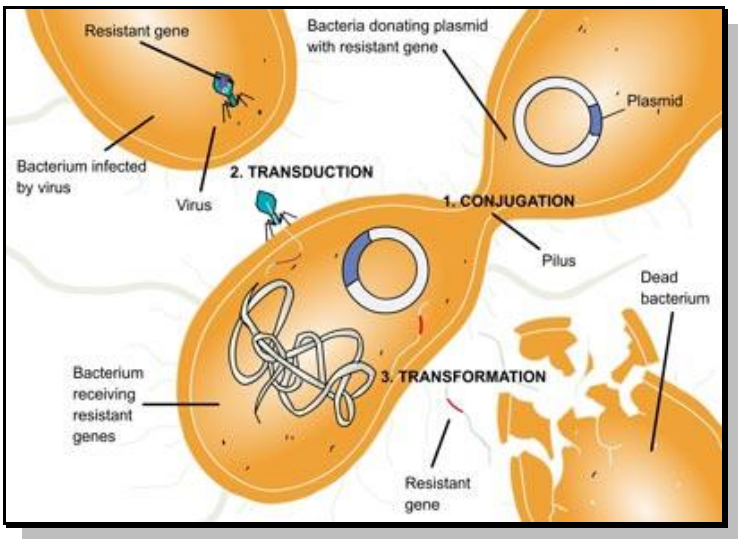

Figure 1. HGT mechanisms in bacteria. 1- Conjugation consists on DNA transfer between two bacteria that are in contact by a protein structure called pilus. 2- Transduction is the transfer of DNA mediated by bacteriophages. Genomic DNA of a phage inserts on the chromosome as a prophage. Later it replies, accidentally and low frequently encapsulating any host DNA (generalized transduction) or DNA near the site of integration of the prophage (specialized transduction), it lyses the cell, and infects a new host cell, in which new DNA can recombine with the chromosome. 3-

Transformation is the uptake of free environmental DNA mediated by proteins encoded in the chromosome of some naturally transformable bacteria.

HGT is mediated by mobile genetic elements. These DNA segments encode enzymes and other proteins that cause movement of DNA within the genome (intracellular mobility) or among bacterial cells (intercellular mobility). Intracellular movements of DNA occur mainly by jumping of transposons among replicons, and since transposons can jump into phages and plasmids, they also can be transferred with them to other cells. Intracellular movement of DNA among prokaryotes can occur by three mechanisms shown in Figure 1: conjugation, transduction and transformation. In transformation, a recipient cell takes up DNA from the environment, such as DNA released from a dead organism. Transduction is the transfer of DNA from one cell to another via a replicating virus.

Although resistance genes can disseminate by HGT mechanism, the vast majority of reports of bacterial gene transfer in the environment involve conjugation (Davison, 1999).

Conjugation

Bacterial conjugation is the transfer of DNA from a donor cell to a receptor cell by a protein complex known as conjugative machinery (figure 2). This process requires both bacteria are in contact and usually it is mediated by plasmids or conjugative transposons. It is the most extended HGT mechanism and that contributes most to the pool of mobile genes in the prokaryotic world (de la Cruz et al., 2000). It has the advantage that the transferred material is a complete autonomous plasmid DNA strand, with its own origin of replication. In transformation or transfection, the transferred DNA fragments may or may not contain complete genes and phenomena of homologous recombination, which decreases efficiency in stable acquiring f transferred genes, must be produced in order to acquire information remains stably in the cell.

Bacterial conjugation, in addition to being a mechanism of genetic variability, plays an important role in infectious diseases spreading virulence determinants, in antibiotic resistance dissemination, in bacterial symbiosis and in xenobiotics degradation. Therefore, it has attracted in recent years a great interest in knowing in detail the activity of the proteins involved in the bacterial conjugation process.

The first indication of the conjugative process was found by Lederberg and Tatum in 1946, some years before Watson and Crick unraveled DNA structure. They found that the *E. coli* K12 strain could act as a donor of genes, obtaining recombinant wild type strains from *E. coli* strains with several auxotrophies, after contacting them with K12.

Later, it was demonstrated that it was a one-way process, and that the ability to carry out was related to the presence of so-called fertility factor F, the F plasmid (Hayes, 1953). For many years, the F plasmid was the only identified conjugative plasmid and it was thought that conjugation was an unusual biological phenomenon. But during the 70s and 80s of the last century, a large number of conjugative plasmids were isolated from gram-negative and gram-positive bacteria and it was recognized as a widespread phenomenon.

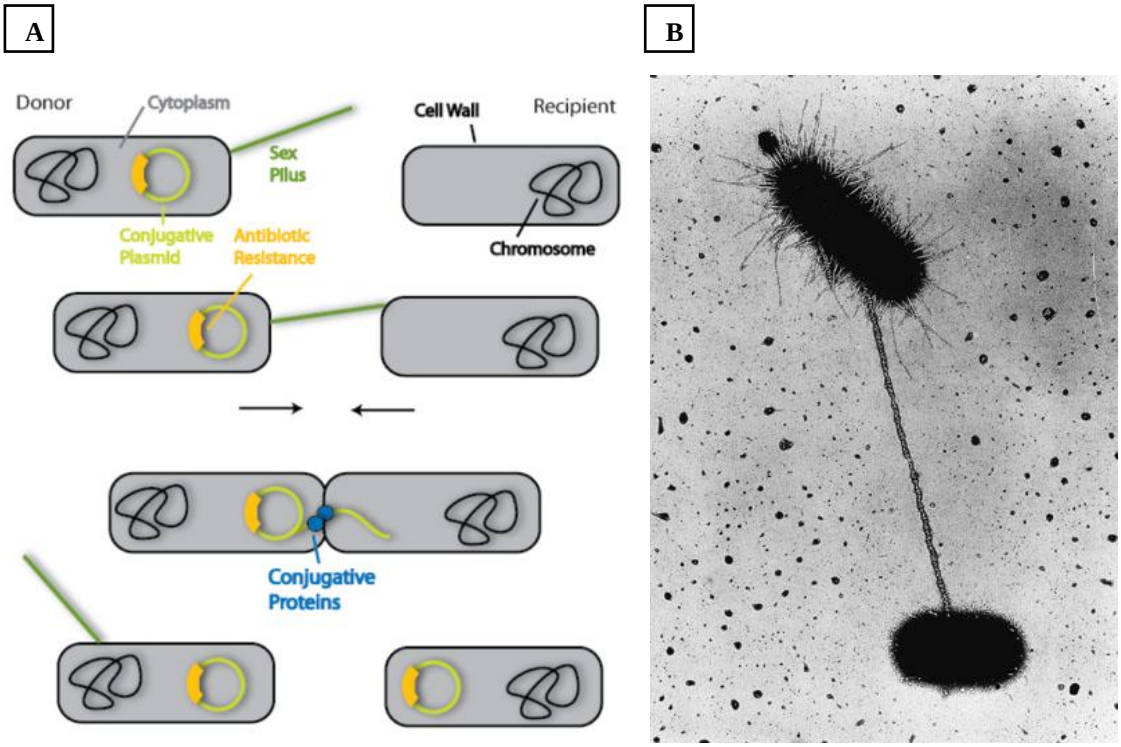

Figure 2. A) Schematic diagram of a bacterial conjugation process involving the transfer of an antibiotic resistance gene. The most important steps are the following: 1- Donor cell produces pilus. 2- Pilus attaches to recipient cell and brings the two cells together. 3- The mobile plasmid is nicked and a single strand of DNA is then transferred to the recipient cell. 4- Both cells synthesize a complementary strand to produce a double stranded circular plasmid and also reproduce pili; both cells are now viable donors. B) Electron microscopic image by Charles C. Brinton, Jr., of a mating pair initially brought together by means of an F pilus.

Plasmids have been classified into incompatibility groups, depending on the specificity of their replication machinery, as plasmids that have the same replication system can not coexist in the same cell. Twenty-six incompatibility groups have been identified for *Enterobacteriaceae* plasmids, 14 groups for *Pseudomonadaceae* plasmids and 18 groups for plasmids of gram-positive bacteria *Staphylococcaceae* (Couturier et al., 1988). The most studied conjugative

plasmids are those of gram-negative bacteria, which belong to the following incompatibility groups: IncF (F, R1, R100), IncW (R388), IncN (pKM101, R46), IncP (RP4, RK2), IncX (R6K), IncI (R64) e IncQ (R1162, RSF1010).

But transfer systems of gram-positive bacteria conjugative plasmids have also been analyzed, such as pAD1, pMV158 or pGO1.

There are other differences among conjugative plasmids, apart from those related to replication. Some are in a wide variety of bacteria, called broad host range plasmids (IncN, IncP, IncW). By contrast, narrow host range plasmids are stable in a limited number of bacterial species (IncF, IncI). The contact between cells can occur through a flexible pilus, which allows bacteria conjugate in liquid medium, a rigid pilus, which causes bacteria to conjugate only on solid medium, or through cell surface proteins, such as gram-positive bacteria.

Some plasmids are self-transmissible, because they are able to produce the whole conjugative machinery to carry out the process, and others that do not produce it complete are mobilizable and they need the presence of a self-transmissible plasmid for conjugation takes place.

In spite of this functional diversity, several conserved features have been described in all conjugative plasmids. All require the synthesis of a conjugative pilus or some other system that mediate intercellular contact to transfer takes place, often carrying functions involved in processing the DNA to be transferred, having some kind of mechanism for the establishment of acquired DNA by the recipient, and usually having linked regulatory systems that set the conditions under which transfer occurs (Zechner et al., 2000).

The functions required for DNA transfer are encoded in conjugative plasmids in a transfer region, *tra*, whose genes are divided into two groups: *dtr* genes (DNA transfer replication), related to DNA processing, and *mpf* genes (mating pair formation), involved in membrane carrier formation.

The transfer region includes a short DNA sequence, called transfer origin (*oriT*), where the process begins and ends. The relaxosome, the nucleoprotein complex that initiates DNA processing, is formed by the *oriT*, a relaxase and one or more accessory proteins.

The relaxase protein specifically cuts in a site of the *oriT*, called *nic*, in the DNA strand to be transferred, remaining covalently attached to cut DNA and presumably religating it at the end of the process (Lanka et al., 1995).

Conjugation current model

The bacterial conjugation process is currently viewed as a mechanism of DNA replication by rolling circle (RCR) system linked to a type IV secretion system of macromolecules (Llosa et al., 2002). This is because, first, relaxases are related to initiation of RCR proteins, with which they share sequence motifs and DNA processing reactions. Moreover, its *oriT* target sequences are structurally related to the RCR origins *oriV*. Otherwise, the set of conjugative proteins that are assembled to form the transmembrane channel, belongs to type IV secretion carriers family (T4SS). This type of carrier is used by *Agrobacterium tumefaciens* to transfer T-DNA to plant cells and also by animal pathogens to inject virulence factors into target cells.

These similarities make the conjugative machinery is schematize as two modules, one that does RCR DNA processing and other, type IV carrier. These two modules are connected by a protein present in conjugative systems, called coupling protein.

A mechanism to DNA transport in bacterial conjugation has been proposed, called "shoot and pump" (Llosa et al., 2002), which consists of two steps (figure 3):

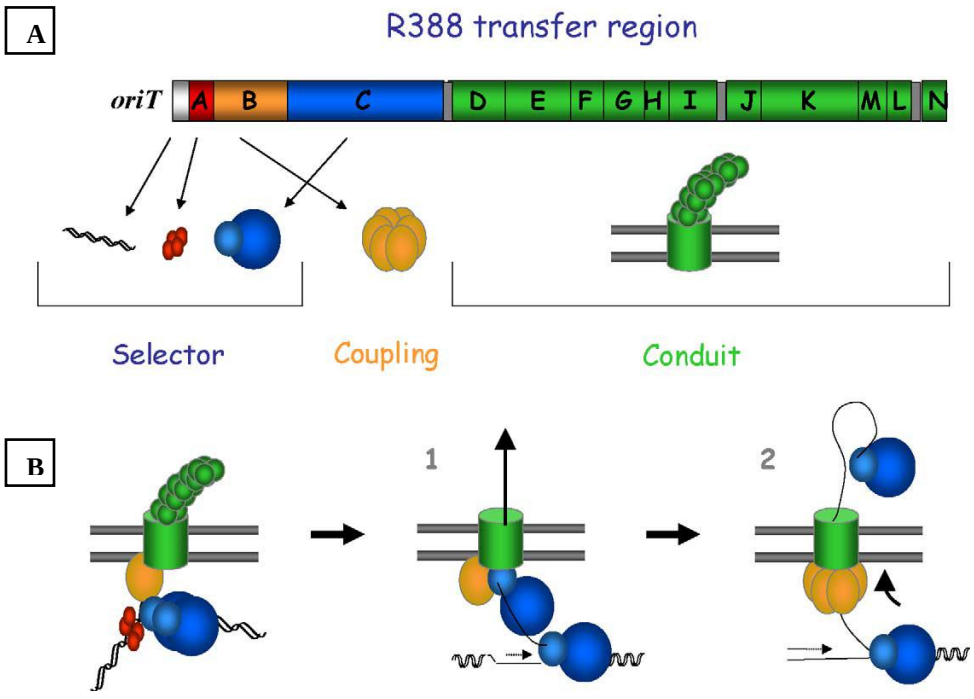

Figure 3. Structure and function of a conjugative DNA transfer system. A) Genetic organization of the transfer region of plasmid R388 and the resulting protein products. The *trw* gene prefix has been omitted for clarity. Proteins are arranged in the indicated functional modules. B) Scheme of the shoot-and-pump model for conjugal DNA transfer. Step 1: the relaxase is secreted through the type IV secretion system,

with the trailing covalently bound DNA strand. Step 2: the remaining DNA is pumped out via the coupling protein (Llosa et al., 2005).

1. The shot or relaxase active transport by the T4SS with the DNA strand attached to the protein.

2. The pump, which is an active movement of the DNA strand that is transferred through the channel. This pumping is produced by the coupling protein.

The various aspects of this model are based on the following experimental evidence:

i) The substrate of the T4SS. The T4SS are essentially protein carriers, as it has been shown that many are used only to secrete proteins like those involved in injecting of virulence factors into mammalian cells. It is therefore reasonable to assume that the transport of DNA in conjugation is a consequence of its covalent attachment to the relaxase, which is the true substrate of T4SS.

ii) The relaxase as pilot protein. In the DNA transfer system of *A. tumefaciens*, VirD2 relaxase plays a functional role in plant cell. MobA relaxase from mobilizable RSF1010 plasmid also seems to pass into recipient cell, judging from indirect studies. Recently the definitive proof of the transportation of the relaxase of a conjugative plasmid (R388 TrwC) to the recipient cell has been published, and this process depends on the T4SS and the coupling protein.

iii) Coupling protein functions as a DNA pump. Based on three-dimensional structure and its similarity to other DNA carriers and the F_1-ATPase, it has been proposed that coupling protein, in addition to functioning as a connector, is involved in DNA active transport, pumping the transferred DNA from donor to recipient cell using the energy produced by ATP hydrolysis. In addition, these proteins show sequence similarity with a family of membrane proteins involved in the transport of DNA, which includes proteins as FtsK and SpoIIIE, for which it has been shown to produce DNA motion. Another indirect evidence is the fact that coupling proteins are associated with T4SS involved in DNA transport, while T4SS from intracellular pathogens such as *Bartonella* and *Brucella*, whose substrates are virulence proteins, lack of protein coupling. The only exception is offered by *H. pylori*, whose T4SS does appear associated with a coupling protein and, to date, it has not been shown to perform DNA transfer. However, it is significant that the coupling protein of *H. pylori* is also able to bind DNA and that in this microorganism two genes that bear sequence similarity and conserved motifs in relaxases have been identified.

iv) The way out of DNA. There is evidence of it for the T-DNA of *A. tumefaciens*. The T-DNA strand (presumably attached to the pilot protein, VirD2) first comes into contact with the coupling protein and then with the cytoplasmic ATPase VirB11. Then, with the T4SS core components; first with those which are in contact with the inner membrane (VirB6 and VirB8) and then with those which are anchored in the outer membrane (VirB9) and with the pilin subunit VirB2.

The general pattern of bacterial conjugation is:

- The conjugative process begins with the formation of the conjugative pair. At this stage, donor and recipient cells come in contact through a protein structure synthesized by the donor cell called conjugative pilus. In the case of the F plasmid, the retraction of this pilus brings closer the cells until they were in direct contact. In this first contact, proteins of Mpf system responsible for pilus formation and conjugative pair stabilization are involved.

- The relaxase binds to *oriT*, cuts the strand that is being transferred and becomes covalently attached to the 5' end. Although the signal that triggers the DNA transfer process is not known, it is thought to be related to the proper cell contact. There is an unwinding of the DNA resulting in single-stranded DNA that is transferred. This helicase activity is performed by the relaxase in some plasmids and in others, by a bacterial DNA helicase. The relaxase attached to the T-DNA (transferred DNA) is passed through the T4SS, bringing the T-DNA. The DNA is initially transported passively by the T4SS, due to transport of the relaxase attached to DNA, and then, actively by pumping the coupling protein.

- Finally, the transferred DNA must be set to the recipient cell. To this end, proteins transported along the DNA from the donor cell and proteins expressed in the recipient cell in early stages are used. The circular ssDNA molecule is converted through the involvement of cellular replication proteins in circular dsDNA and later supercoiled.

Once transferred DNA, cells disaggregate and express in recipient cell the plasmid genes that lead to the surface exclusion, thus preventing the entry of new copies of the same plasmid. The recipient strain can act then as a donor, restarting a new cycle of transfer.

Antibiotic resistance spreading

Antibiotics are one of the most apparent success stories of modern medicine and have saved the lives of countless people that suffered from bacterial infections. However, the use of antibiotics has also led to the emergence of AbR in bacteria. The development of AbR and the distribution of resistant bacteria throughout the biosphere are caused by a decades-long selection process through the application of antibiotics in humans, animals and plants (Davies et al., 2010).

AbR carried by common human bacterial pathogens has reached a global dimension (Boucher et al., 2009). The emergence of antibiotic resistant pathogens is a major threat to human health as therapeutic options for treating infections by AbR bacteria are increasingly limited. The incidence of AbR bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, *Klebsiella*, and others has skyrocketed over the past two decades (figure 4). Moreover, the success of resistant organisms contributes to the constant accumulation in the bacterial world of genetic platforms and vehicles able to efficiently recruit and spread novel resistance genes.

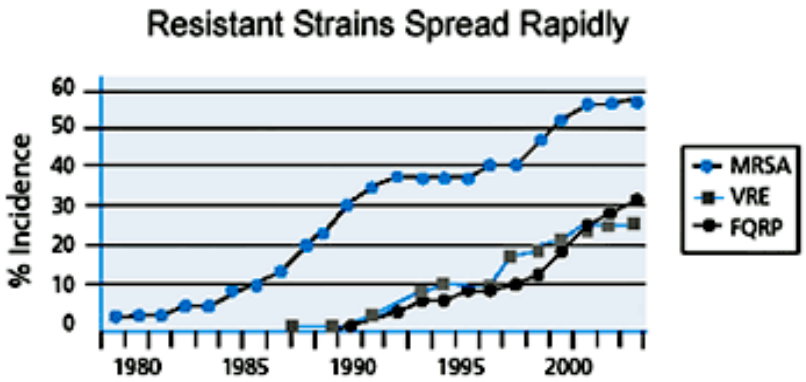

Figure 4. Incidence (percentage) of resistant strains. MRSA: methicillin-resistant *Staphylococcus aureus*; VRE: vancomycin-resistant Enterococci; FQRP: floroquinolone-resistant *Pseudomonas aeruginosa*. (Centers for Disease Control and Prevention).

Indeed, infections due to AbR bacteria are a major cause of morbidity and mortality in both hospitals and the community throughout the world (Hawkey et al., 2009). Each year, these infections kill nearly 100,000 U.S. hospital patients and are increasingly affecting healthy people as well. In addition, the number of new antibiotics being developed has plummeted. While 16 new antibiotics were approved between 1983 and 1987, only two have been approved since 2008 (figure 5).

Antibiotics are becoming less effective due to over-prescription and improper use (up to half of antibiotic use is unnecessary or inappropriate) as well as bacteria's natural ability to evolve and develop resistance to antibiotics. Prudent use of antibiotics is a logical and necessary step to decelerate resistance development, but this will not completely stop the spread of antibiotic resistance among human pathogens (World Health Organization, 2000). Treating these resistant bugs costs the U.S. health care system an estimated $21 billion to $34 billion annually. Drug companies now are shifting their research dollars to developing drugs that treat chronic conditions, such as diabetes and high blood pressure. These drugs are less challenging to bring to market than antibiotics from a regulatory standpoint and are much more lucrative because they are used for years, rather than days or weeks, as antibiotics are. In 1990, there were nearly 20 pharmaceutical companies with large, strong and active antibiotic Research and Development programs. Today, there are just two, and only a small number of companies have more limited programs (Infectious Diseases Society of America).

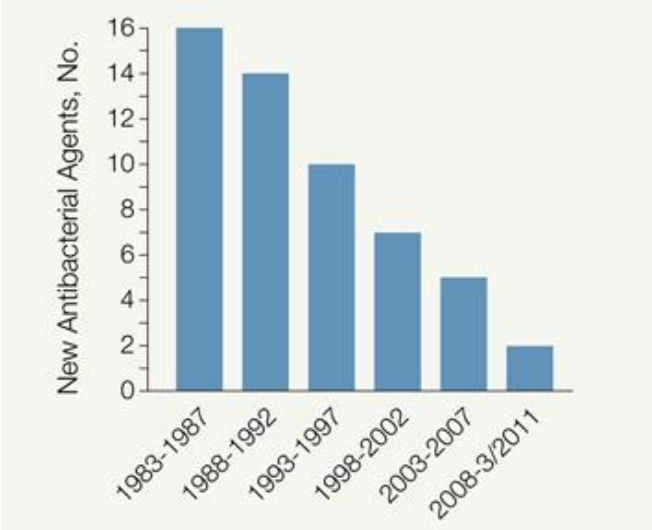

Figure 5. New antibacterial agents approved by the FDA in the United States, 1983–2011, per 5-year period (Infectious Diseases Society of America).

In this context, the need for new strategies, instead of new antibiotics, arises with the purpose of solving the problem of the AbR spreading. We (Baquero et al., 2011) have proposed to treat bacterial populations and their environment as an ecosystem, being this ecosystem then subject to prevent the acquisition or limit AbR dissemination. This kind of strategies are called eco-evo. Eco-evo strategies are those leading to interventions that aim not necessarily to kill resistant organisms, but rather to prevent their emergence and evolution, or even to re-

establish the antibiotic-susceptible populations. The purpose is to combat resistance not only in infected patients, but rather in a whole population composed of infected and non-infected people alike, as occurs in hospitals, nurseries, or elderly-care facilities, as well as in general hot environments ("resistance reactors"), facilitating the evolution of AbR. By extension, other environments that can be successfully treated are farms, fish factories and eventually water effluents. Drugs with this properties are called eco-evo drugs. Thus, this new type of drugs will act not necessarily to cure the individual patient, but to "cure" specific environments from AbR, and to prevent or weaken the evolutionary possibilities of the biological elements involved in AbR. An example of eco-evo drugs are COINS (conjugation inhibitors). This project aims to find new bacterial systems having this property, to prevent AbR propagation.

Antecedents

In the recipient cells, conjugation can be inhibited by different mechanisms: restriction systems, CRISPRs, entry exclusion systems or incompatibility, among others.

Restriction modification systems are used by bacteria to protect themselves from foreign DNA, cleaving doubled stranded strange DNA (with different methylation pattern), such as conjugative plasmids (Tock et al., 2005), by restriction endonucleases.

CRISPRs are Clustered Regularly Interspaced Short Palindromic Repeats that acts as interference RNA in eukaryotes and can limit conjugation (Marraffini et al., 2008). In a few words, an exogenous DNA sequence, for instance from a virus or a conjugative plasmid, is processed and incorporated among these repeats and when that sequence is detected again, it is recognized by CRISPRs and degraded.

Entry exclusion system consists of a change on the surfaces of plasmid containing cells which inhibits the transfer of related plasmids (Garcillán-Barcia et al., 2008).

Incompatibility groups include plasmid which are closely related and share similar replication functions, leading to the exclusion of one or the other plasmid if they are present in the same cell.

The first two mechanisms are usually encoded in the chromosome, while the last two are plasmid mechanisms to inhibit the entry of related plasmids. Apart from these mechanisms, little is known about inhibiting conjugation ways in the recipient cell.

Our group developed some time ago a method for high-throughput analysis of conjugation that found that unsaturated fatty acids were inhibitors of bacterial conjugation (Fernández-López et al., 2005). This method was then used to check for host genes in the recipient cell involved in conjugation (Pérez-Mendoza et al., 2009), by using as recipients all the mutants in the Keio collection (Baba et al., 2006) and a collection of 20,000 random mini-Tn10::Km insertion mutants in *E. coli* strain DH5α, concluding that lab strains of *Escherichia coli* like DH5α have no non-essential genes that play an essential role in conjugation.

Objective

In this context, the main purpose of this work is to investigate on some of the natural barriers that bacteria impose to the propagation of plasmids, mainly of broad host range plasmids, such as R388, which can disseminate in widely different bacteria, in order to find new targets to control AbR dissemination before infection.

Perhaps these natural barriers, once their mechanisms of action are known, can be used by humans, after due manipulation, synthesis, etc., to control plasmid dissemination. If plasmid dissemination can somehow be controlled, this will lead to a new class of therapeutic drugs that will principally target, not the patients themselves, but the ecosystems in which these plasmids are transmitted to the human pathogens (Baquero et al., 2011).

As no genes were founded in the genome of the recipient strain that inhibits conjugation (Pérez-Mendoza et al., 2009), our next step is searching for inhibition genes of conjugation in plasmid DNA of recipient strains, objective of this project (another ongoing work in our group consists of looking for genes in donor cell involved in conjugation). To do this, we will screen a set of enterobacterial plasmids clinically isolated in search of new mechanisms which can inhibit, not only the entry of related plasmids, but also of unrelated ones, in order to use them in the future to control spreading of AbR genes.

Results and discussion

HTC assay

Brief description of the method

High throughput conjugation (HTC) assay allows us to analyze many samples at once. Briefly, two donor strains containing plasmid pSU2007::Tn*lux* or pOX38::Tn*lux* (in which the 5-gene *lux* operon is under the control of the *lac* promoter) and pUC18::*lacI*^{q}, is mixed with cultures containing recipients and conjugation allowed to occur for four hours on solid medium (figure 7). As explained in figure 6, cultures not expressing light contain recipients potentially affecting conjugation (i.e., affecting transfer to or establishment in the recipient). Both types of recipients are relevant to the objectives of this project.

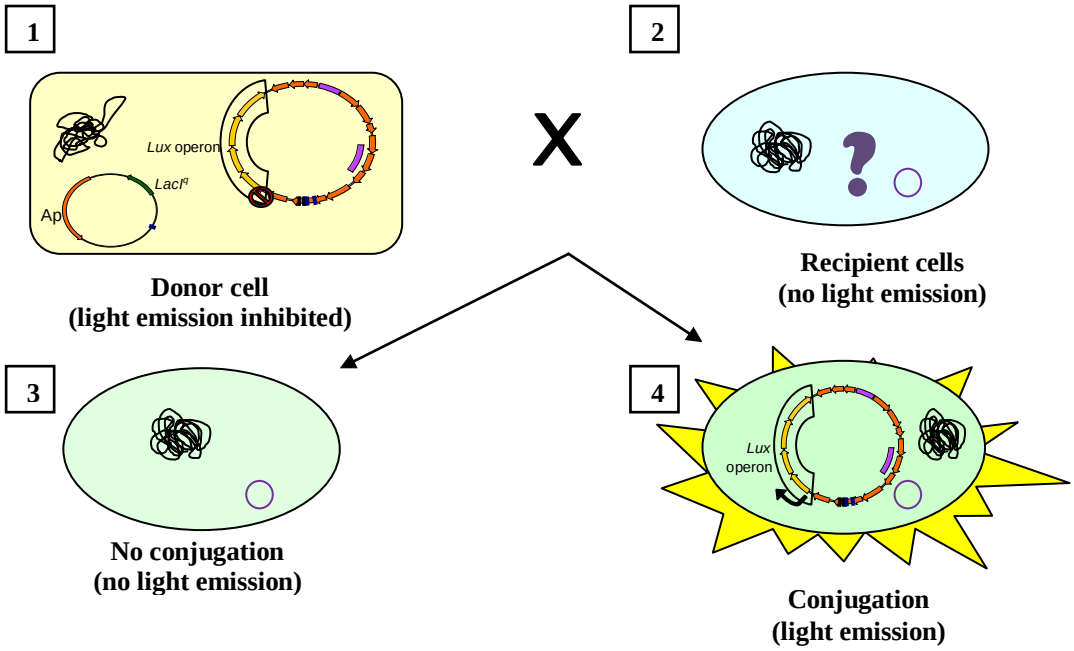

Figure 6. Schematic diagram of the HTC assay. 1- Donor cells UCDPM1 and UCDPM2 contain pSU2007::*lux* (IncW) or pOX38::*lux* (IncF) respectively, and pUC18::*lacI*^{q}. *Lux* operon is under the control of *lac* promoter, which is repressed by the *lacI* gene in the pUC18, a non-conjugative plasmid. 2- Recipient cells have different natural plasmids, which are tested to inhibit conjugation. 3- When donor and recipient cells are mixed and no conjugation occurs, light emission remains inhibited because donor plasmid do not move into recipient cell. 4- When conjugation occurs, conjugative plasmid with *lux* operon move into recipient cell, where LacI repressor is not present, so light is emitted (chromosomal copy of *lacI* is not sufficient to repress *lux* expression, it is needed a multicopy *lacI*^{q}). Image courtesy of Irene del Campo.

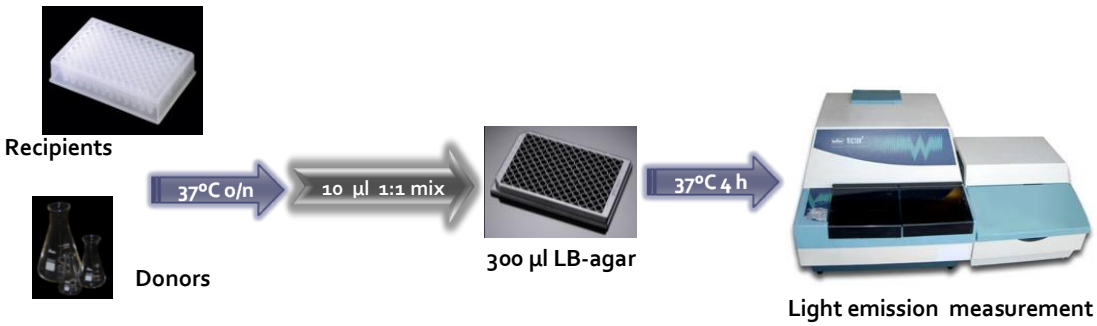

Figure 7. Experimental scheme of HTC assays. Recipient and donor cells (table 1) grown overnight at 37ºC were mixed in a 1:1 ratio and 10 µL of each resulting conjugation mixture were spotted into 96 well black microtiter plates containing 300 µl LB agar, and conjugation was allowed to proceed for 4 h at 37ºC. Luminescence was measured as arbitrary light units (ALU) using a microplate luminometer.

Strains used

All the strains, controls and problem collections used in HTC assay are described in table 1 and table 2.

	Strain/Control	Characteristics
Donors	**UCDPM1**	*E. coli* CSH53 containing pSU2007::*lux* (conjugative R388 derivative) and pUC18::*lacI*^{q} (lux system repressor)
	UCDPM2	*E. coli* CSH53 with pOX38::*lux* (conjugative F derivative) and pUC18::*lacI*^{q} (lux system repressor)
Recipients	**DH5α**	Positive control, good recipient ability, *E. coli* DH5α without plasmids
	pSU5024	Negative control to UCDPM1/positive control to UCDPM2, *E. coli* DH5α containing a plasmid with *eex*_R388 gene, entry exclusion system of R388
	Empty	Negative control, without recipient cells
	Background	Negative control, without donor and recipient cells
	Problem collections	Wild type and transconjugants in lab strains with different natural plasmids clinically isolated to be tested

Table 1. Strains and controls used in HTC assay and their characteristics.

Collection	Characteristics
Reference	57 isolates in lab strains containing plasmids representative of each type of relaxase
Barcelona	16 clinical isolates in *E. coli* HB101
Goteborg	10 conjugative mercury-resistant plasmids isolated from marine environments, 8 in *E. coli* lab strains and 2 in *Pseudomonas putida* lab strains
Valdecilla	13 original isolates from Holspital de Valdecilla (Santander) and 23 transconjugants in *E. coli* J53
Ramón y Cajal	15 extended spectrum beta-lactamase (ESBL) producing Enterobacteriaceae from Spain (1988-2008) and 66 transconjugants harbouring ESBL-coding plasmids in different lab strains
Sant Pau &Santa Creu	37 wild type isolates and 58 transconjugants in lab strains
Austrian	19 original *E. coli* isolates from urine cultures of Swedish women that suffered from community-acquired uncomplicated urinary tract infections carrying an unknown number of plasmids and 30 transconjugants in *E. coli* DH5α

Table 2. Recipient collections used in HTC assay and their characteristics.

HTC assay validation

Appropriated controls support using the HTC assay (figure 8).

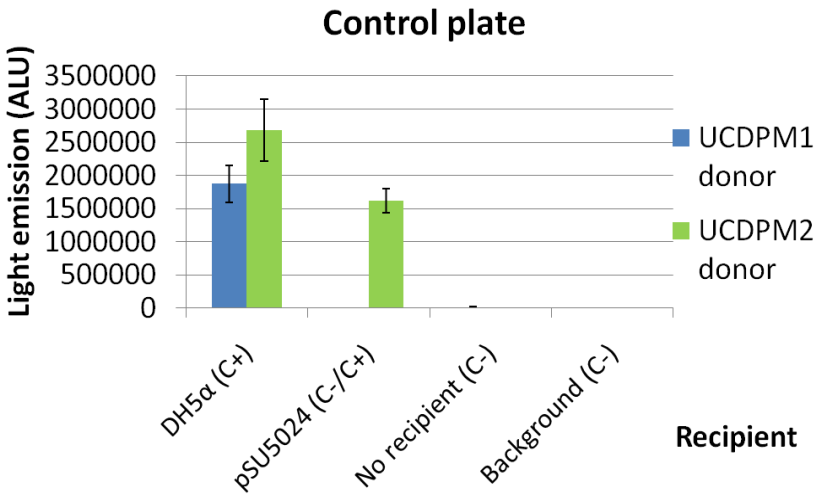

Figure 8. Graph representing a control assay of both donors, UCDPM1 and UCDPM2, tested under HTC conditions with different recipients. 1- Recipient *E. coli* DH5α was used as positive control of conjugation due to its good recipient ability (Pérez-Mendoza et al., 2009). 2- Recipient with plasmid pSU5024, which overproduces Eex_R388, entry exclusion system of R388, was used as control of poor recipient ability of related plasmids, so it behaves as a negative control to UCDPM1 and as a positive control to UCDPM2 (Garcillán-Barcia et al., 2008). 3- Without recipient, there should not be conjugation nor light emission, unless repression system of donor light emission was damaged. 4- Without cells, LB-agar background do not emit light. ALU: Arbitrary Light Units.

HTC results

In tables 3-9 are represented the light emission percentage referred to the positive control (*E. coli* DH5α) of the 344 plasmid-containing recipients analyzed, what is related to conjugation frequency as explained before. Some of the recipients used were in their original host strain (wild type) while others were hosted in lab strains. In the latter case, an step of conjugation from wt to different lab strains was previously done to test the recipient ability due to its plasmids.

Recipient (Transconjugant)	UCDPM1 (% Light emission)	UCDPM2 (% Light emission)	Recipient (Transconjugant)	UCDPM1 (% Light emission)	UCDPM2 (% Light emission)
R#R388	4.2	7.3	R#pSN254	24	4.1
R#pSa	5.5	6.5	R#R55	0.35	7.1
R#pKM101	110	145	R#Rts1	16	7.4
R#Pwwo	3.6	4.9	R#pKLC102	75	78
R#pBi709	6.4	7.5	R#CloDF13	90	120
R#F	3.5	7.1	R#pAM373	2.1	8.5
R#R100	44	65	R#RSF1010	39	4.7
R#pKDSC50::Tn1	104	140	R#pIE1115	18	6.8
R#pED208	8.6	4.4	R#pIE1130	74	112
R#pTET3	111	148	R#pNAC2	79	157
R#pGA2	16	28	R#pIGWZ12::Km	89	83
R#pN3	62	84	R#pK214	9.8	5.5
R#RP4	39	5.4	R#pUB110	5.2	7.8
R#R751	38	4.6	R#pCTX-M3	30	66
R#pUO1	4.2	9.2	R#R387	26	4.4
R#pB10	43	19	R#R711b	27	8.7
R#pEST4002	51	19	R#R394	13	5.4
R#R64	23	144	R#pIP55	28	8.2
R#pET46	61	45	R#pEL60	13	69
R#pTC-F14	74	80	R#R446	3.5	2.8
R#pRAS3.1	81	92	R#pSU316	16	3.4
R#pRAS3.2	86	108	R#pIP1100	23	6.9
R#R6K	7.6	13	R#RIP55	41	4.1
R#pOLA52	30	5.2	R#R7K	1.2	2.1
R#pRA3	33	28	R#pIE321	1.4	11
R#ColE1	61	39	R#PRL443	2.7	13
R#pAsal3	132	151	R#pB10	98	132
R#R27	67	61	R#pIE522	2.5	18
R#R478	29	4.4	DH5 α	100	100

Table 3. Percentage of light emission referred to DH5α, measured after 4 h conjugation of UCDPM1 and UCDPM2 to recipients from the "Reference" collection. Values represented are the mean of six different results.

Recipient (Transconjugant)	UCDPM1 (% Light emission)	UCDPM2 (% Light emission)
B#1	19	6.9
B#2	9.2	3.5
B#3	33	8.3
B#4	30	5.8
B#5	42	7.5
B#6	24	5.0
B#7	32	9.0
B#8	25	9.3
B#9	4.0	3.1
B#10	23	9.6
B#11	25	8.1
B#12	25	8.6
B#13	3.3	4.4
B#14	47	42
B#15	7.3	5.9
B#16	25	2.5
DH5 α	100	100

Table 4. Percentage of light emission referred to DH5α, measured after 4 h conjugation of UCDPM1 and UCDPM2 to recipients from the "Barcelona" collection. Values represented are the mean of six different results.

Recipient (Transconjugant)	UCDPM1 (% Light emission)	UCDPM2 (% light emission)
G#203	0.08	0.10
G#237	1.6	2.4
G#599	110	104
G#600	146	135
G#601	128	127
G#602	134	132
G#603	129	132
G#604	19	0.23
G#605	22	0.63
G#606	49	35
DH5α	100	100

Table 5. Percentage of light emission referred to DH5α, measured after 4 h conjugation of UCDPM1 and UCDPM2 to recipients from the "Goteborg" collection. Values represented are the mean of six different results.

Recipient		UCDPM1 (% light emission)		UCDPM2 (% light emission)	
Wild type	Transconjugant (J53)	Wild type	Transconjugant (J53)	Wild type	Transconjugant (J53)
V#1	V#2	0.26	3.1	0.01	0.28
V#3	V#4	1.0	23	0.01	0.44
	V#5		21		0.68
	V#6		19		0.40
V#7	V#8	13	45	0.13	0.41
	V#9		43		0.19
V#10	V#11	5.2	32	0.04	0.29
	V#12		30		0.20
V#13	V#14	1.1	33	0.01	0.48
V#15	V#16	2.1	50	0.02	0.55
	V#17		47		0.63
V#18	V#19	1.3	47	0.07	0.31
	V#20		45		0.44
V#21	V#22	2.3	8.9	0.03	0.12
V#23	V#24	3.1	48	0.04	0.33
	V#25		53		0.39
V#26	V#27	0.53	55	0.05	0.59
V#29	V#30	12	49	0.29	1.8
	V#31		49		1.8
	V#32		55		1.7
V#33	V#34	2.3	51	0.23	0.48
V#36	V#37	0.09	47	0.04	0.16
	V#38		28		0.07
DH5α	DH5α	100	100	100	100

Table 6. Percentage of light emission referred to DH5α, measured after 4 h conjugation of UCDPM1 and UCDPM2 to recipients from the "Valdecilla" collection. Values represented are the mean of six different results.

Recipient (Wild type)	UCDPM1 (% Light emission)	UCDPM2 (% Light emission)	Recipient (Wild type)	UCDPM1 (% Light emission)	UCDPM2 (% Light emission)
RC#9	0.43	2.2	RC#61	0.45	8.4
RC#13	14	1.8	RC#62	0.45	5.0
RC#15	0.27	1.7	RC#63	1.2	0.42
RC#19	0.22	0.89	RC#65	30	4.5
RC#35	22	3.5	RC#71	6.1	3.0
RC#54	2.6	2.5	RC#79	0.43	5.0
RC#55	1.8	20	RC#80	0.05	0.05
RC#56	0.35	8.1	DH5α	100	100
Recipient (Transconjugant)	**UCDPM1 (% Light emission)**	**UCDPM2 (% Light emission)**	**Recipient (Transconjugant)**	**UCDPM1 (% Light emission)**	**UCDPM2 (% Light emission)**
RC#3	21	3.3	RC#41	11	5.0
RC#4	32	8.1	RC#42	9.6	3.9
RC#5	0.16	0.81	RC#43	12	2.7
RC#6	15	3.3	RC#44	24	1.9
RC#7	12	1.9	RC#45	27	2.2
RC#8	13	8.4	RC#46	16	2.9
RC#10	3.8	4.7	RC#47	26	2.9
RC#11	0.57	2.5	RC#48	0.38	2.9
RC#12	7.6	4.9	RC#49	26	4.2
RC#14	26	3.3	RC#50	11	4.2
RC#16	0.31	1.2	RC#51	21	4.6
RC#17	0.68	8.3	RC#52	0.15	0.44
RC#18	1.8	16	RC#53	34	4.4
RC#20	17	3.1	RC#57	34	5.0
RC#21	17	0.09	RC#58	23	2.4
RC#22	20	4.6	RC#59	7.4	6.0
RC#23	23	4.1	RC#60	25	3.7
RC#24	20	3.1	RC#64	16	2.3
RC#25	3.9	6.3	RC#66	20	3.2
RC#26	30	4.0	RC#67	11	2.8
RC#27	35	2.4	RC#68	46	2.4
RC#28	25	4.3	RC#69	38	4.6
RC#29	5.8	3.8	RC#70	15	5.8
RC#30	32	4.7	RC#72	3.5	4.5
RC#31	23	3.9	RC#73	23	2.4
RC#32	23	4.4	RC#74	25	3.6
RC#33	17	4.9	RC#75	21	4.8
RC#34	2.8	5.1	RC#76	22	2.0
RC#36	0.34	2.9	RC#78	16	2.4
RC#37	0.15	1.6	RC#81	17	4.6
RC#38	1.9	4.5	RC#82	16	3.9
RC#39	4.0	3.0	RC#83	11	4.9
RC#40	22	2.5	RC#84	21	2.7

Table 7. Percentage of light emission referred to DH5α, measured after 4 h conjugation of UCDPM1 and UCDPM2 to recipients from the "Ramón y Cajal" collection. Values represented are the mean of six different results.

Recipient (Wild type)	UCDPM1 (% Light emission)	UCDPM2 (% Light emission)	Recipient (Wild type)	UCDPM1 (% Light emission)	UCDPM2 (% Light emission)
S#S.marcescens D	0.14	0.02	S#90D	10	3.0
S#E. coli D	0.23	0.04	S#93D	2.1	2.8
S#24D	3.0	5.7	S#100D	0.07	0.02
S#27D	1.1	1.2	S#115D	0.59	0.08
S#30D	5.8	7.3	1	6.0	0.11
S#33D	3.0	2.6	2	0.01	0.00
S#34D	3.8	4.0	3	1.2	0.00
S#40D	3.5	0.76	4	3.8	0.20
S#44D	0.76	0.58	6	0.71	0.00
S#49D	3.5	5.8	8	0.27	0.01
S#55D	6.5	11	9	0.70	0.04
S#64D	9.2	11	10	0.06	0.00
S#65D	4.8	3.9	11	1.9	0.00
S#66D	1.0	2.0	12	1.7	0.00
S#72D	0.71	0.12	13	1.1	0.01
S#74D	1.5	0.74	3.08	1.6	0.01
S#75	16	14	5.08	1.1	0.00
S#76D	1.3	0.93	7.08	2.1	0.03
S#79D	4.3	7.0	DH5α	100	100
Recipient (Transconjugant)	**UCDPM1 (% Light emission)**	**UCDPM2 (% Light emission)**	**Recipient (Transconjugant)**	**UCDPM1 (% Light emission)**	**UCDPM2 (% Light emission)**
S#1	25	4.6	S#84	26	4.3
S#2	1.9	1.9	S#88	32	5.2
S#3	1.5	2.5	S#89	24	15
S#4	2.3	7.8	S#91	13	5.5
S#5	13	9.7	S#92	27	4.2
S#6	14	11	S#94	16	19
S#7	12	15	S#95	20	8.4
S#8	1.2	2.0	S#96	18	15
S#9	12	9.0	S#97	13	12
S#10	15	12	S#98	13	13
S#11	14	11	S#99	10	12
S#12	8.1	7.1	S#101	0.05	0.01
S#13	10	6.3	S#102	10	6.1
S#14	22	9.1	S#103	17	14
S#15	13	9.9	S#104	0.07	0.03
S#16	12	13	S#105	10	12
S#18	13	15	S#106	0.35	0.09
S#19	13	9.9	S#107	16	16
S#20	14	16	S#108	16	22
S#25	3.2	6.0	S#109	20	22
S#28	0.50	0.37	S#110	17	15
S#61	15	13	S#111	18	16
S#71	4.6	0.11	S#112	18	13
S#75	6.4	1.4	S#113	16	13
S#78	18	2.0	S#114	12	11
S#80	3.3	4.6	S#Tc1	0.02	0.00
S#81	5.3	2.7	S#Tc3	7.5	0.09
S#82	3.3	2.5	S#Tc11	9.0	0.08
S#83	23	5.1	S#Tc14	9.5	0.02

Table 8. Percentage of light emission referred to DH5α, measured after 4 h conjugation of UCDPM1 and UCDPM2 to recipients from the "Sant Pau & Santa Creu" collection. Values represented are the mean of six different results.

Recipient		UCDPM1 (% light emission)		UCDPM2 (% light emission)	
Wild type	Transconjugant (DH5α)	Wild type	Transconjugant (DH5α)	Wild type	Transconjugant (DH5α)
A#3031	A#13081	0.16	0.41	0.02	3.8
A#3033	A#12110	0.32	1.2	0.07	7.9
A#3065	A#11130	1.8	1.1	0.01	0.07
	A#11150		0.39		0.05
	A#12031		0.81		0.03
	A#12041		2.4		0.04
A#3097	A#1050	0.47	49	0.04	13
A#3100	A#12134	1.4	1.4	0.18	6.9
	A#12135		16		5.1
A#3175	A#13111	0.01	1.1	0.04	9.3
A#3201	A#1009	0.99	20	0.20	39
	A#1029		2.3		198
	A#1030		3.8		100
	A#11054		71		15
	A#11057		20		42
A#3271	A#13101	0.02	0.04	0.26	0.05
A#3315	A#11003	0.26	0.42	0.01	0.06
A#3323	A#11010	0.11	71	0.00	0.15
A#3632	A#11084	0.02	0.01	0.01	0.04
	A#11100		0.82		0.05
A#3707	A#12061	0.32	60	0.05	49
A#3718	A#1040	0.12	31	2.9	39
A#3899	A#13022	86	0.06	48	0.07
	A#13023		0.10		0.14
A#3942	A#12033	0.19	0.11	0.04	0.30
	A#12034		0.92		0.54
A#3989	A#13091	0.01	0.06	0.00	0.08
A#4138	A#1100	0.73	13	0.01	1.6
A#4371	A#13061	5.0	4.2	0.20	6.5
A#4393	A#13031	0.11	0.83	0.08	8.8
DH5α	DH5α	100	100	100	100

Table 9. Percentage of light emission referred to DH5α, measured after 4 h conjugation of UCDPM1 and UCDPM2 to recipients from the "Austrian" collection. In purple are highlighted those plasmid-containing derivatives of DH5α whose light emission is less than 1% with both donors. Values represented are the mean of six different results.

Summary of HTC results

Of the 344 clinical isolates used as recipients in the HTC experiment using two K12 donors containing either plasmid R388 or plasmid F (two unrelated conjugative plasmids), 73 % of the wt recipients in the seven collections (84 in total) inhibited plasmid conjugation of both donors by more than 95 %, whereas only 17 % of the transconjugants in lab strains (260 in total) inhibited conjugation by more than 95 % (table 10).

These results indicate that clinical isolates of E. coli, and probably also environmental isolates, contain means to avoid conjugation, something that a disarmed E. coli K12 does not. Although some of these mechanisms are expected to be known (restriction systems, for example), we expect to find new mechanisms, since this is an aspect of plasmid biology that has been insufficiently analyzed.

In addition, as empty lab strains like DH5α are good recipients (Pérez-Mendoza et al., 2009) and inhibition is produced with both donors, these results also suggest that most wt clinical isolates have systems to inhibit the entry of related and unrelated plasmids encoded in their genomes (such as restriction systems or CRISPRs, for example), while a minority of these systems are located in plasmids. These latter systems encoded in plasmids are particularly important for us, since they will be, by definition, "mobile" and therefore could be implemented in widely different bacterial backgrounds, so they are the searched mechanisms

Recipient collection	Wild type (% inhibition)		Lab strains (% inhibition)	
	>95%	>99%	>95%	>99%
Reference	- (0)	- (0)	3.5 (2)	0.0 (0)
Barcelona	- (0)	- (0)	13 (2)	0.0 (0)
Goteborg	- (0)	- (0)	20 (2)	10 (1)
Valdecilla	77 (10)	23 (3)	4.4 (1)	0.0 (0)
Ramón y Cajal	40 (6)	13 (2)	17 (11)	3.0 (2)
Sant Pau & Santa Creu	76 (28)	30 (11)	19 (11)	8.6 (5)
Austrian	89 (17)	74 (14)	50 (15)	40 (12)
TOTAL	**73 (61)**	**36 (30)**	**17 (44)**	**7.7 (20)**

Table 10. Summary of the HTC screening. Wild-type and transconjugants (in lab strains) isolates of the collections used as recipients are considered by separate. Percentage of isolates inhibiting conjugation over 95 and 99% is shown. The hyphen represents absence of wild type recipients in the collection. In brackets, total number of isolates represented by the percentage.

Re-assaying HTC hits

Since *E. coli* DH5α was the empty lab strain used as a control of good recipient ability because no non-essential genes were found that play an essential role in conjugation (Pérez-Mendoza et al., 2009), transconjugants in this strain that showed the highest inhibition of conjugation were the recipients chosen to re-assay their conjugation frequencies (CF). Thus, inhibition shown will be caused by plasmids contained in DH5α.

Recipients with lowest ALU also exhibited low CF

Some recipients colored in purple from Table 9 that showed the highest inhibition were selected for standard conjugation assays to verify their ability to inhibit conjugation of pSU2007 (table 11). Thus, we eliminated the false positives due to spurious decreased luminescence emission. While positive control used as recipient showed a CF per recipient of 0.74, CFs from selected recipients are less than 10,000 fold using the same donor. These results are similar to those observed in HTC assay, indicating that this high throughput experiment to measure conjugation was comparable to conjugation frequencies calculated by standard conjugation assays selecting transconjugants and recipients depending on their AbR after conjugation.

Recipient	Replicon	Relaxase type	CF (recipient)
DH5α (C+)	-	-	0.74
A#11084	RepF, RepI1, RepX	MOB_{P51}, MOB_{F12}	$3.55 \cdot 10^{-5}$
A#12033	RepF	MOB_{P51}, MOB_{P12}	$1.04 \cdot 10^{-5}$
A#12034	RepF	MOB_{P51}, MOB_{P12}	$2.05 \cdot 10^{-5}$
A#13091	-	MOB_{P51}, MOB_{qu}	$3.14 \cdot 10^{-5}$
A#13101	-	MOB_{P51}, MOB_{P3}	$1.74 \cdot 10^{-5}$

Table 11. Conjugation frequencies of selected recipients, measured after standard assays of conjugation with pSU2007 plasmid. The value represented is the mean of four different results.

Low CF vs donor killing

To be sure this inhibition was broad range, besides R388 (IncW) and F (IncFI) used in HTC assay and R388 again in standard conjugation assay, some positive hits will be retested using some other unrelated plasmids: R100 or R1 (IncFII), pKM101 or pN3 (IncN), R751 (IncP) and R6K (IncX), depending on plasmid and recipient AbR.

Thus, we rechecked the CF of the recipient A#13091 as an example using *E. coli* CSH53 RifR donors containing plasmids of different incompatibility groups (table 12) to prove

that chosen recipients inhibited conjugation of unrelated plasmids. In this experiment, we realized that donor frequencies (in purple) considerably diminished in conjugation assays with A#13091 compared to those with the positive control (DH5α). This suggested that the selected recipient promoted inhibition in donor growth, indirectly causing CF decrease. As A#13091 was in DH5α strain, this inhibition system had to be encoded in its plasmids.

Recipient	Donor	Donors/mL	Recipients/mL	Transconjugants/mL	CF (donor)	CF (recipient)
A#13091	R388 (IncW)	$1.0 \cdot 10^{2}$	$4.0 \cdot 10^{7}$	$< 1 \cdot 10^{2}$	< 1.0	$< 2.5 \cdot 10^{-6}$
	R751 (IncP)	$1.0 \cdot 10^{2}$	$6.7 \cdot 10^{7}$	$< 1 \cdot 10^{2}$	< 1.0	$< 1.5 \cdot 10^{-6}$
	pN3 (IncN)	$3.3 \cdot 10^{1}$	$3.3 \cdot 10^{7}$	$6.7 \cdot 10^{1}$	2.0	$2.0 \cdot 10^{-6}$
	R100 (IncFII)	$4.7 \cdot 10^{2}$	$3.3 \cdot 10^{7}$	$< 1 \cdot 10^{2}$	< 0.2	$< 3.0 \cdot 10^{-6}$
	F (IncFI)	$3.3 \cdot 10^{1}$	$3.3 \cdot 10^{7}$	$1.0 \cdot 10^{2}$	3.0	$3.0 \cdot 10^{-6}$
DH5α	R388 (IncW)	$4.3 \cdot 10^{8}$	$1.3 \cdot 10^{8}$	$1.3 \cdot 10^{5}$	$3.0 \cdot 10^{-4}$	$1.0 \cdot 10^{-3}$
	R751 (IncP)	$5.4 \cdot 10^{8}$	$1.0 \cdot 10^{8}$	$7.3 \cdot 10^{5}$	$1.4 \cdot 10^{-3}$	$7.1 \cdot 10^{-3}$
	pN3 (IncN)	$3.4 \cdot 10^{8}$	$1.6 \cdot 10^{8}$	$8.0 \cdot 10^{5}$	$2.3 \cdot 10^{-3}$	$4.9 \cdot 10^{-3}$
	R100 (IncFII)	$1.9 \cdot 10^{8}$	$1.5 \cdot 10^{8}$	$1.9 \cdot 10^{5}$	$1.0 \cdot 10^{-3}$	$1.3 \cdot 10^{-3}$
	F (IncFI)	$3.3 \cdot 10^{7}$	$1.0 \cdot 10^{8}$	$1.2 \cdot 10^{6}$	$3.7 \cdot 10^{-2}$	$1.2 \cdot 10^{-2}$

Table 12. Conjugation frequencies of recipient A#13091, measured after standard assays of conjugation with different plasmids. Donor and recipient strain are CSH53 (RifR) and *E. coli* DH5α (NxR) respectively.

Growth inhibition: colicins

Most selected recipients inhibit DH5α growth

As the inhibition of growth system was encoded in plasmids, it could not be, for example, an antibiotic synthesis mechanism, because they are encoded in big clusters of genes. However, it was likely to be a small system, such as colicin synthesis operons, that appears in colicinogenic plasmids, like ColE1 or CloDF13. These operons encode genes for the synthesis and resistance to colicins, proteins of 40-80 kDa secreted by some strains of *E. coli* lethal to related strains. Table 13 shows a relation of several colicin types, their target receptor proteins where they attached, their import proteins that permit the entry in the target cell and mechanisms by which colicins kills target bacteria (forming pores, degrading DNA, degrading RNA…).

Colicin	Receptor	Import	Cytotoxicity
Colicins[a]			
Group A			
A	BtuB	OmpF, TolABQR	Pore
E1	BtuB	TolC, TolAQ	Pore
E2-E7-E8-E9	BtuB	OmpF, TolABQR	DNase
E3-E4-E6	BtuB	OmpF, TolABQR	16S RNase
E5	BtuB	OmpF, TolABQR	tRNA-(Y-H-N-D)-specific RNase
K	Tsx	OmpF, OmpA, TolABQR	Pore
N	OmpF	OmpF, TolAQR	Pore
U	OmpA	OmpF, TolABQR	Pore
Cloacin DF13	IutA	OmpF, TolAQR	16S RNase
Group B			
B	FepA	TonB-ExbBD	Pore
D	FepA	TonB-ExbBD	tRNA-(R)-specific RNase
Ia-Ib	Cir	TonB-ExbBD	Pore
M	FhuA	TonB-ExbBD	Degradation of the C55 phosphate-linked peptidoglycan precursors
5–10	Tsx	TolC, TonB-ExbBD	Pore

Table 13. Cell envelope proteins required for reception and translocation steps of different colicins and mode of action (Cascales et al., 2007).

In order to analyze selected recipient ability to inhibit donor growth, an inhibition of growth test (version 1) was done. As observed in figure 9, every tested recipient formed an inhibition halo around it, but negative control (DH5α) and A#13101 recipient. This result suggests the presence of an inhibition of growth mechanism, such as colicins synthesis. This plasmid mechanism seems to synthesize any compound able to inhibit *E. coli* DH5α growth that is secreted to the medium and diffuses more or less depending on its size, forming different halo sizes.

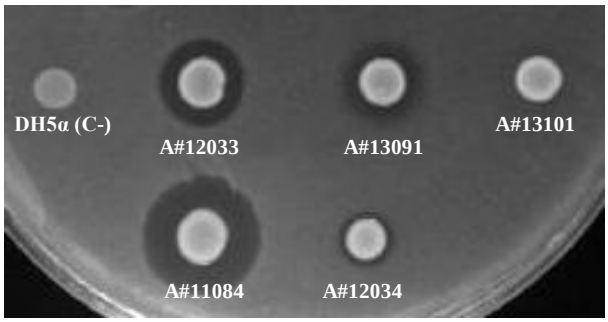

Figure 9. Photograph of a plate with *E. coli* DH5α acting as a marker to test growth inhibition by selected recipients of "Austrian" collection (2 µl concentrated bacteria). *E. coli* DH5α was used as negative control.

Since A#13101 recipient did not apparently inhibit donor growth so its low CF seems to be due to inhibition in conjugation itself instead of donor killing, its CF was re-checked using unrelated plasmids.

Recipient	Donor	CF (donor)	CF (recipient)	% CF (donor)	% CF (recipient)
A#13101	R388 (IncW)	$1.9 \cdot 10^{-5}$	$2.7 \cdot 10^{-6}$	0.12	0.01
	R751 (IncP)	$6.6 \cdot 10^{-3}$	$6.5 \cdot 10^{-4}$	16	0.60
	pKM101 (IncN)	$1.2 \cdot 10^{-3}$	$6.9 \cdot 10^{-5}$	1.9	0.06
	R6K (IncX)	$1.5 \cdot 10^{-4}$	$1.6 \cdot 10^{-5}$	19	0.70
	R1 (IncFII)	$3.4 \cdot 10^{-3}$	$2.8 \cdot 10^{-3}$	13	4.7
	F (IncFI)	$1.1 \cdot 10^{-2}$	$7.8 \cdot 10^{-4}$	9.2	0.70
DH5α	R388 (IncW)	$2.6 \cdot 10^{-2}$	$5.4 \cdot 10^{-2}$	100	100
	R751 (IncP)	$3.7 \cdot 10^{-2}$	$1.1 \cdot 10^{-1}$	100	100
	pKM101 (IncN)	$6.3 \cdot 10^{-2}$	$1.1 \cdot 10^{-1}$	100	100
	R6K (IncX)	$6.9 \cdot 10^{-4}$	$1.6 \cdot 10^{-3}$	100	100
	R1 (IncFII)	$2.5 \cdot 10^{-2}$	$4.6 \cdot 10^{-2}$	100	100
	F (IncFI)	$1.9 \cdot 10^{-1}$	$2.7 \cdot 10^{-1}$	100	100

Table 14. Conjugation frequencies of recipient A#13101 per donor and recipient, measured after standard assays of conjugation with different plasmids. Donor strain is CSH53 (RifR). The value represented is the mean of three different results.

Although low CFs are observed (table 14), comparing CF percentage referred to positive control per donor and per recipient, an increase in CF per donor is observed in A#13101 regarding to CF per recipient. As CF is calculated as the number of transconjugants divided by number of donors or recipients, this increment means that A#13101 plasmids could be inhibiting donor growth yet. To confirm this hypothesis, a new inhibition of growth test (version 2) was done, this time using filtrated supernatant of selected cultures.

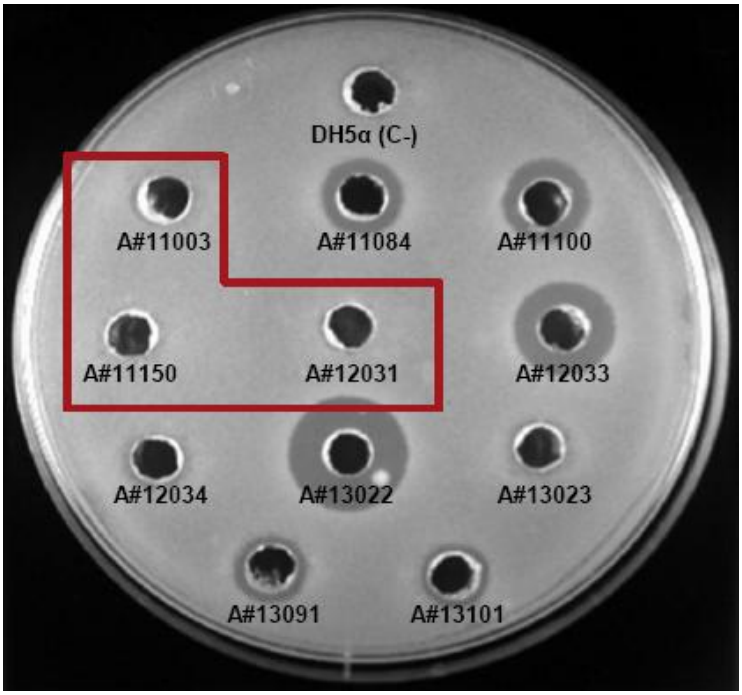

Figure 10. Growth inhibition test. Photograph of a plate cultured with *E. coli* DH5α. Filtered supernatant cultures of the selected recipients of the "Austrian" collection were spotted. Boxed in red, recipients that did not inhibit DH5α growth.

Results shown in figure 10 are congruent with the presence of colicin synthesis mechanisms. An inhibition halo was formed around filtrated supernatant of most recipients, but three, A#11003, A#11150 and A#12031. These non-killer recipients were also those lacking MOB_{P51} and MOB_{C11} relaxases, typically present in colicinogenic plasmids such as ColE1 or CloDF13 (table 15).

Recipient	Relaxase type
A#11003	MOB_{F12}, MOB_{P3}
A#11084	MOB_{F12}, MOB_{P51}
A#11100	MOB_{P12}, MOB_{P51}
A#11150	MOB_{P12}
A#12031	-
A#12033	MOB_{P51}, MOB_{C12}, MOB_{P12}
A#12034	MOB_{P51}, MOB_{C12}, MOB_{P12}
A#13022	MOB_{P51}, MOB_{C11}, MOB_{C12}, MOB_{P12}, MOB_{P3}
A#13023	MOB_{P51}, MOB_{C11}, MOB_{C12}, MOB_{P3}
A#13091	MOB_{qu}, MOB_{P51}
A#13101	MOB_{P51}, MOB_{P3}

Table 15. Relaxase types of *E. coli* DH5α transconjugants with > 99% inhibition of conjugation (Alvarado et al., manuscript in preparation). In purple, recipients without MOB_{P51} or MOB_{C11} relaxase type, present in colicinogenic plasmids such as ColE1or CloDF13 respectively.

Plasmid selection

Some plasmids are broad-range conjugation inhibitors, without affecting cell growth

Recipients A#11150 and A#12031, that did not produce inhibition halo, were rechecked for their ability to reduce the conjugation of different conjugative plasmids. A#11003 have too many AbR so it could not be used in standard conjugation assays.

Viable donor cells did not vary regarding the positive control (plasmid-lacking DH5α as recipient), a clear indication that the plasmids contained in A#11150 and A#12031 are not inhibiting cell growth. Significantly, their CFs per donor and per recipient drastically dropped regardless of the conjugative plasmid contained in the donor cells (table 16). No transconjugants were obtained with all the donors but F, unlike the positive control.

This means that both recipients are able to inhibit conjugation itself of several unrelated plasmids, due to a mechanism encoded in their plasmids.

Recipient	Plasmid in donor	CF (donor)	CF (recipient)
DH5α	R388 (IncW)	$3.1 \cdot 10^{-2}$	$5.8 \cdot 10^{-2}$
	R751 (IncP)	$6.4 \cdot 10^{-2}$	$1.4 \cdot 10^{-1}$
	pN3 (IncN)	$7.4 \cdot 10^{-2}$	$2.1 \cdot 10^{-1}$
	R100 (IncFII)	$7.5 \cdot 10^{-3}$	$1.3 \cdot 10^{-2}$
	F (IncFI)	$4.3 \cdot 10^{-1}$	$8.2 \cdot 10^{-1}$
A#11150	R388 (IncW)	$< 3.4 \cdot 10^{-7}$	$< 1.6 \cdot 10^{-7}$
	R751 (IncP)	$< 3.3 \cdot 10^{-7}$	$< 1.9 \cdot 10^{-7}$
	pN3 (IncN)	$< 2.5 \cdot 10^{-7}$	$< 1.9 \cdot 10^{-7}$
	R100 (IncFII)	$< 2.3 \cdot 10^{-7}$	$< 1.8 \cdot 10^{-7}$
	F (IncFI)	$4.8 \cdot 10^{-5}$	$1.5 \cdot 10^{-5}$
A#12031	R388 (IncW)	$< 3.2 \cdot 10^{-7}$	$< 1.4 \cdot 10^{-7}$
	R751 (IncP)	$< 2.4 \cdot 10^{-7}$	$< 1.1 \cdot 10^{-7}$
	pN3 (IncN)	$< 2.3 \cdot 10^{-7}$	$< 1.7 \cdot 10^{-7}$
	R100 (IncFII)	$< 2.6 \cdot 10^{-7}$	$< 1.3 \cdot 10^{-7}$
	F (IncFI)	$6.4 \cdot 10^{-5}$	$1.4 \cdot 10^{-5}$

Table 16. CF of selected recipients in conjugation assays with donors containing different plasmids (mean of three independent experiments). Donor strain is *E. coli* CSH53 (RifR).

In order to see how many plasmids these recipients had, we extracted their plasmid DNA (figure 11). Line 3 is a size control: plasmid RP4 (60 kb) and R388 (34 kb) in the same strain. Lines 1 and 2 correspond to selected recipients. A plasmid of similar size appears in both recipients, with a size significantly bigger than 60 kb.

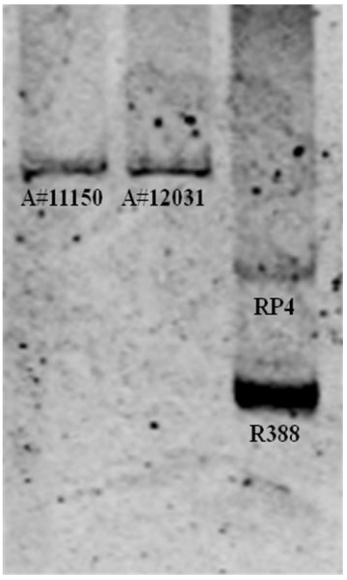

Figure 11. Plasmid visualization in 0.7 % agarose gel after DNA extraction by alkaline lysis. Image courtesy of Andrés Alvarado.

Since A#11150 and A#12031 are transconjugants coming from the same original isolate, A#3065 (see table 9), they have apparently a plasmid with the same size and CFs observed with both recipients are practically identical, our supposed inhibitory plasmid to be tested could be the same.

Conclusions

Conclusions drawn from obtained results were the following:

1. Most clinical isolates analyzed have mechanisms that inhibit plasmid conjugation at least indirectly.

2. In a minority, these mechanisms are encoded by plasmids that can be transferred to a lab *E. coli* strain.

3. Some of these plasmids encode genes for the synthesis of colicins, which inhibit the growth of the donor strain.

4. We found at least one plasmid that inhibited, not the cell growth, but the conjugation of several plasmids. Therefore, we have implemented a HTS method to detect new mechanisms of inhibition of bacterial conjugation.

Once a mechanism to broadly inhibit plasmid conjugation is characterized, we have to think about how to use it to control AbR propagation, depending on the type of system found. If we find, for instance, a mechanism to synthesize some kind of inhibiting compound or protein that can interfere with any level of conjugation system, conserved among conjugative plasmids, we could consider about administrate it in the possible reservoirs where resistance dissemination takes place.

Nevertheless, AbR is a natural biological phenomenon with many facets that are still poorly understood: what are important reservoirs of AbR? How do resistant and non-resistant bacteria interact in these reservoirs? Which conditions promote the evolution and transfer of resistance? Expanding our knowledge on these aspects will provide novel leads to combat the emergence of AbR.

Future research

Sequentially, the next steps programmed to identify the mechanism responsible for the broad-range inhibition of conjugation are the following:

1. Transformation test of A#11150 and A#12031 with some plasmids in order to discard general systems capable of indirectly inhibit conjugation of several plasmids breaking strange double stranded DNA, such as restrictions systems or CRISPRs, that could be encoded in plasmids too.

2. Complete DNA sequencing of A#11150 and A#12031 plasmids. The sequences will be analyzed in a bioinformatic search of possible inhibiting mechanisms.

3. Genetic analysis of inhibition mechanisms to identify the genes or genes responsible for the unknown inhibition mechanism. To achieve this goal, we will use two methods, both in progress:

    a. Direct cloning. Plasmid DNA from selected recipients will be extracted, randomly fragmented and repaired. Fragments larger than 1 kbp will be cloned in pSU19 and used to transform *E. coli* DH5α (figure 12).This library composed of random fragments of plasmid DNA will be subjected to the HTC assay. Clones of interest will be those in which light emission is inhibited because its insert is responsible for inhibiting conjugation. In order to identify the genes present in the cloned fragment, clones of interest will be subjected to sequencing using primers complementary to the ends of pSU19 plasmid.

    b. Random mutagenesis by transposition. If the searched mechanism is complex and is encoded by several genes, it is possible that the fragments we clone do not contain the whole functional elements. In this case: (a), we will mutate plasmid DNA from selected recipients by electroporating a suicidal plasmid containing the minitransposon 10 KmR (mini-Tn10::*Km*). Selecting KmR transformants, we will only have those recipients with the Tn inserted in their genomes or plasmids. We will conjugate their plasmids to another strain in order to select KmR mutants, with the Tn insertion in their conjugative plasmids (we already know that our inhibiting plasmids are conjugative because they were previously conjugate into DH5α strain). This library constituted of transpositional mutants of plasmid DNA will be subjected to another cycle of

HTC assay. Mutant of interest are those which light emission is induced due to mutation of the fragment involved in conjugation repression. In order to identify the genes present in the mutated fragment, mutant of interest will be subjected to sequencing to identify the causal genes. We will follow the same procedure that it was used to search for Tn insertions in DH5α genome affecting R388 conjugation (Pérez-Mendoza et al., 2009), that is, cutting plasmid DNA using an endonuclease and religating it to use then primers complementary to the Tn ends in order to amplify and sequence the adjacent region to the Tn mutated.

4. If appropriate (depending on the results), the mechanisms of inhibition will be investigated by isolating the implicated proteins or protein complexes and analyzed biochemically.

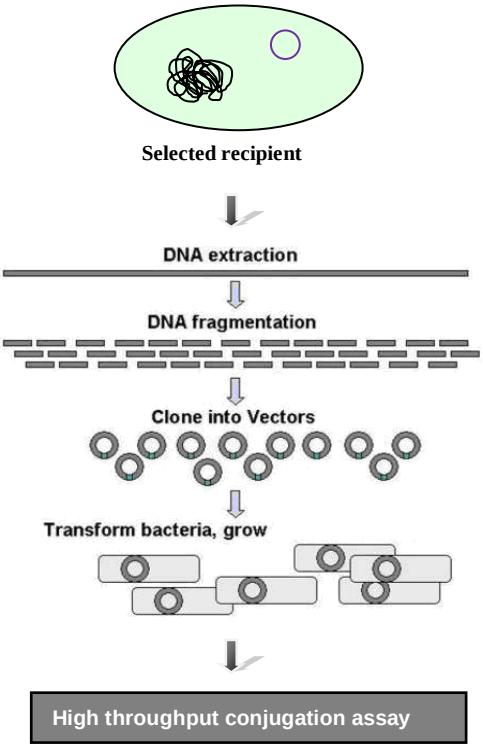

Figure 12. Schematic diagram to construct libraries with random fragments from plasmid DNA. 1- Plasmid DNA of selected recipient is purified. 2- Sonication of DNA is perform to fragment it. 3- 1 kpb fragments and over are purified and repaired to clone into opened vectors. 3- Bacteria is transformed and grown to select then those which have incorporated pSU19 with an insert (white CmR colonies). 4- Finally, colonies selected are used as new recipients in a new high throughput conjugation assay to find a fragment with inhibition of conjugation properties.

Materials and methods

Bacterial strains and plasmids

A derivative of *Escherichia coli* strain CSH53 [*ara D(lac-pro) strA thi (Ô80ΔlacI)*] harbouring plasmid pSU2007::*Tnlux* and pUC18::*lacI*^{q} was used as UCDPM1 donor in conjugation experiments (Fernández-López, 2005). The same strain CSH53 harbouring plasmid pOX38::*Tnlux* and pUC18::*lacI*^{q} was used as UCDPM2 donor. A collection of clinic isolates with different AbR plasmids and transconjugants in lab strains of clinic isolated plasmids were used as recipients to test its conjugation ability. *E. coli* strain DH5α [F- *supE44 lacU169 (Ô80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (Hanahan, 1983) was used as positive control of recipient capacity and to construct both libraries. Strains containing plasmid pSU5024 (Fernández-López, 2005), which overproduces Eex_R388, were used as controls of poor recipient ability. Plasmid pSU19 was used to do the transformation test and to clone fragments of plasmid DNA from sonication. Plasmid pLOF-Km (Herrero et al., 1990), that contains mini-Tn*10*::Km, was used to generate random mutants in *E. coli* by direct electroporation of strain DH5α. When appropriate, antibiotics were added at the following concentrations: ampicillin sodium salt (Ap; 100 μg/ml), kanamycin sulphate (Km; 25 μg/ml), nalidixic acid (Nx; 20 μg/ml) cloramphenicol (Cm; 25 μg/ml), rifampin (Rif; 50 μg/ml), tetracycline (Tc; 10 μg/ml), streptomycin (Sm; 20/300 μg/ml), trimethoprim (Tp; 20 μg/ml).

High throughput conjugation assay

A high throughput assay, based on visible light emission (Fernández-López, 2005), was carried out to test conjugation activity. A single colony of the donor strain was grown at 37°C in LB with Km and Ap overnight. Individual colonies of recipients were inoculated in 96 deep well plates (Axigen) and grown overnight at 37°C with agitation. 200 μl of the donor strain were added to the wells of the recipient plates, each containing 200 μl of an individual recipient. A copy of each recipient was generated for storage before adding the donor strain. For the experiments under surface mating conditions, 10 μl of each resulting conjugation mixture were spotted into 96 well black microtiter plates (Thermo Electron Corporation) containing 300 μl LB agar and conjugation was allowed to proceed for 4 h at 37ºC. Donor cells contained conjugative plasmid pSU2007::Tn*lux* and non-conjugative pUC18::*lacI*^{q} (so expression of the lux operon was completely repressed and donor bacteria were non-luminescent). Upon conjugation, pSU2007::Tn*lux*, but not pUC18::*lacI*^{q}, moves to the recipient cell, resulting in expression of luminescence in transconjugants. Luminescence was measured as arbitrary light units (ALU) using a microplate luminometer (Victor2 from Perkin Elmer). Light emission obtained with empty DH5a recipient was considered 100 %. All measurements are referred to it.

Standard conjugation experiments

Donor and recipient strains, grown to stationary phase, were washed in LB and mixed in a 1:1 ratio. Mating mixtures were resuspended in 15 µl LB and deposited onto 24-well plates (Nunc) with 900 µl LB-agar into each well. Plates were incubated for 1 h at 37°C. Then, they were resuspended in 1 mL liquid LB and diluted to select transconjugants on plates supplemented with appropriate antibiotics. The transfer frequency was expressed as the number of transconjugants per output recipient or donor. Transfer rates were normalized to the wt strains (DH5α) and expressed as a percentage.

Inhibition of growth test

Version 1. *Escherichia coli* DH5α was plated uniformly in LB-agar and 2 µl of concentrated selected recipients and DH5α (negative control) were spotted onto it. Plates were moved into 37ºC chamber to grow them overnight and be photographed by Chemidoc™ (Bio-Rad).

Version 2. *Escherichia coli* DH5α (negative control) and selected recipients were grown overnight without any antibiotic that can interfere the test. Cultures were centrifuged at 13,000 rpm for 3 minutes and supernatant was sterilized by filtration with 0.45 µm pore size membrane. *Escherichia coli* DH5α was plated uniformly in LB-agar and let dry to make then some holes in the medium. 100 µL of filtered supernatant were added into these holes and subjected to diffusion for 2 hours at 4ºC. Plates were then moved into 37ºC chamber to grow them overnight and be photographed by Chemidoc™ (Bio-Rad).

Plasmid visualization (Kado et al., 1981)

Control and problem strains were streaked on LB-agar plates and incubated overnight at 37°C. Bacteria were transferred into 2 ml of L-broth, incubated overnight at 37°C without shaking and transferred 1.5 ml into an Eppendorf tube to centrifuge at 13,000 rpm for 10 minutes at room temperature. Supernatant was removed, leaving the bacterial pellet as dry as possible. Bacterial pellet was resuspended in 20 µl of Buffer 1 (E-buffer: 50mM Tris / 1mM EDTA, pH 8). 100 µl of freshly prepared Buffer 2 (Lysis – Mix: 3% SDS and 50 mM Tris, pH 12.6 adjusted with NaOH) were added, to incubate then 27 minutes at 58°C. 100 µl freshly prepared solution of high quality Acid Phenol/Chlorophorm (1:1) were added and mix gently until the solution is completely white. It was centrifuged at 13,000 rpm for 30 minutes at room temperature and the supernatant was removed by gently aspiration and add 90 µl supernatant to 20 µl sample buffer. It was stored on ice for 19 minutes and 90 µl of the DNA – preparation were concentrated and added on a 0.7 % agarose gel subjected then to electrophoresis 15 min at

50 V and 2 h at 100 V. Gel was stained with Et-Br (0.5 mg/ml) 20 min, destained with distilled water 20 min and photographed under UV-light.

Transformation test (in progress)

Electrocompetent cells of selected recipients and *E. coli* DH5α as positive control were prepared according to the instructions of the manufacturer and stored at -80°C. Plasmid DNA at first from pSU19 (CmR) was purified by using Plasmid MiniPrep kit (ATP Biotech). Electroporation was carried out in an electro cell manipulator apparatus (BioRad) and cells were immediately suspended in 1 ml LB medium and incubated at 37°C for 1 h. Appropriate dilutions were plated on LB-agar with Cm to select transformed cells and transformation frequencies were calculated per total cells, referring to positive control (100%).

Plasmid sequencing (in progress)

Plasmid DNA of selected recipient was extracted using Plasmid MaxiPrep kit (Sigma), according to manufacturer instructions. Plasmid DNA was sequence via Illumina Sequencing Service (University of Cantabria).

Construction of libraries with random fragments from plasmid DNA (in progress)

Plasmid DNA of selected recipient was extracted using Plasmid MaxiPrep kit (Sigma), according to manufacturer instructions, in order to construct randomized genetic libraries. For this, approximately 20 μg of DNA were concentrated 1:10 and randomly fragmented by sonication in a non-refrigerated Bioruptor device (Diagenode). Seven cycles of 30" sonication at highest power with 30" intervals were carried out. Obtained fragments were visualized in 1% agarose gels stained with RedSafe (ChemBio) and those around 1.5 kpb were recovered with ATP Gel/PCR extraction kit (ATP Biotech Inc.). The amount recovered was measured using Nanodrop and approximately 1 μg of DNA was blunted using the (Illumina Corp. Kit Ref.). The product was purified again with ATP Gel/PCR extraction kit (ATP Biotech Inc.). The DNA repaired was ligated into pSU19 (CmR) linearized with SmaI (Fermentas), according to manufacturer indications (Illumina Corp.). *E. coli* DH5α was transformed with ligation product and plated in LB-agar supplemented with Cm and X-gal (60 μg/ml). White colonies were selected and subjected to PCR procedures with Taq DNA polymerase (Promega) to ensure that they contained a DNA fragment of the expected size. PCR-positive clones were grown overnight in LB+Cm and were used as recipients in a new conjugation screening. Those clones with inhibited conjugation were selected to extract its plasmid DNA with Plasmid MiniPrep Kit (ATP Biotech Inc.). These clones with a possible inhibition of conjugation system were

sequenced via Macrogen services (South Korea) in direct and complementary directions to find the responsible mechanism.

Construction of mutant libraries by transposition (in progress)

Selected recipient electroporation to generate mini-Tn*10*::Km insertions was carried out in an electro cell manipulator apparatus (BioRad). Electrocompetent cells were prepared according to the instructions of the manufacturer and stored at -80°C. For electroporation, cells were thawed on ice, mixed with pLOFKm DNA (0.3–0.5 μg of DNA per ml of cell suspension) and transferred to a 0.2 cm electrode gap chilled cuvette. A pulse of 2.5 kV/cm field strength, 6.8 ms time and 129 Ω set resistance was applied, cells were immediately suspended in 1.0 ml LB medium and incubated at 37°C for 1 h. Appropriate dilutions were plated on LB-agar with Km. In order to choose bacteria with insertions into their plasmids, an additional step of conjugation to another recipient strain with different AbR was carried out, then selecting new transconjugants on selective media with both antibiotics (Km and Ab). Those recipients with verified insertions in their plasmids were used in a new conjugation screening, this time to select recipients with incremented conjugation activity, due to inactivation by transposition of a potential mechanism of conjugation inhibition. After choosing recipients with higher light emission, plasmid DNA was purified using Plasmid MiniPrep Kit (ATP Biotech Inc.) to start the mapping of transposon insertion sites. Fifty nanograms of plasmid DNA from each KmR mutant was digested with *Csp6I* endonuclease. Five ng of the digested genomic DNA was religated in 20 μl final volume and incubated overnight at 16°C. Five μl of the ligation reaction were used as template for an inverse PCR reaction using oligonucleotides Tn10IR (CTGATGAATGTTCCGTTGCG) and Tn10Km (ACCTGGAATGCTGTTTTCCC). The amplified PCR-products were purified from agarose gels and both ends sequenced using Tn10IR and Tn10Km primers. DNA sequence homology search was performed with BLAST program from NCBI (Altschul et al., 1997) to determine the position of the transposon insertion.

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