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Review

Regulation of conjugative transfer of plasmids and integrative conjugative elements

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ABSTRACT

Horizontal gene transfer has been recognized as one of the principal contributors to bacterial evolution and diversification. One of the mechanisms involved in this process is conjugative transfer of plasmids and Integrative Conjugative Elements (ICEs). Plasmids and ICEs often encode traits beneficial for bacterial survival in specific environments, or for the establishment of symbiosis or pathogenesis, in addition to genes allowing conjugative transfer. In this review, we analyze the mechanisms that regulate the expression of conjugative transfer genes. For traits such as antibiotic or metal resistance, the compounds involved may induce conjugative transfer directly, while symbiosis and pathogenesis are modulated by quorum-sensing and/or signal molecules released by the host. However, multiple layers of regulation are usually involved in modulating transfer. In addition to the plasmid-encoded regulatory elements, conjugation seems to be regulated by what we have labeled as the "internal environment", defined by the interaction between the host chromosome and the plasmids or ICEs. Another regulatory level depends on the "external environment", which affects conjugative transfer due to the composition and conditions of the community.

1. Introduction

For many years bacterial diversification and evolution were thought to be due to mutations, which are inherited vertically through cellular division. This is partially true, although, with the aid of molecular biology and genomics, a substantial contribution of horizontal gene transfer (HGT) events to this phenomenon has been uncovered (Koonin, 2016). This started with the discovery of conjugation in the 1950s, in addition to transformation and transduction. At present, the literature contains experimental evidence for conjugation in many microorganisms (Reviewed in Aminov, 2011). The wealth of information provided by the sequencing of genomes also shows ample

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bioinformatic evidence for a high proportion of horizontally transferred genetic information in bacteria (Ochman et al., 2000).

Plasmids can vary greatly in size, copy number and in the traits they encode (Thomas and Summers, 2008). They may contain genes conferring resistance to antibiotics and metals, the utilization of carbon compounds, virulence or symbiosis determinants (Dejonghe et al., 2000; Heuer and Smalla, 2012; Top et al., 1995; Wyrsch et al., 2016). These features are also present in Integrative Conjugative Elements (ICEs) (Johnson and Grossman, 2015; Wozniak and Waldor, 2010).

Plasmids and ICEs are often able to perform conjugative transfer, by themselves, or with the aid of other genomic elements. Conjugative transfer requires the expression of transfer (*tra*) genes involved in DNA transfer and replication (Dtr) and in Mating pair formation (Mpf). Dtr genes are required for processing the DNA with a relaxase, which cuts the DNA molecule at the *nic* site of the origin of transfer (*oriT*), and remains attached to the single strand of DNA, which will be transferred responding to an undefined signal (Koraimann and Wagner, 2014). This complex, together with other proteins, makes up the relaxosome, which directs the strand to the type 4 secretion system (T4SS), a multiprotein complex located in the membrane of the cell. The Mpf encodes the genes that are responsible for synthesis of the T4SS. The relaxase, with the DNA strand attached, transfers to the recipient cell through the pore formed by the T4SS, finally the plasmid needs to be established in the recipient, with the help of ssDNA binding, anti-restriction and SOS inhibition proteins to generate stable transconjugants. (Alvarez-Martinez and Christie, 2009; Ding and Hynes, 2009; Frost et al., 2005; Koraimann and Wagner, 2014). It is interesting to note that expression of transfer genes is induced in a subset of the bacterial population, maybe diminishing the negative effects of the associated energy cost (Koraimann and Wagner, 2014).

Conjugative plasmids and ICEs from Gram-negative bacteria usually carry all the structural genetic elements required to perform transfer (an *oriT* site, Dtr and Mpf genes) (Frost et al., 2005). In Gram-positive bacteria, there are two different systems (Goessweiner-Mohr et al., 2014). The first one, which is widely distributed, is similar to that present in Gram-negative bacteria, where a single stranded DNA is transferred through a T4SS (Goessweiner-Mohr et al., 2014; Li et al., 2013; Wisniewski and Rood, 2017). In the second one, present in *Streptomyces*, the double stranded plasmid molecule is transferred through the walls of the hyphae, suggesting that conventional Mpf genes are not required, indicating important differences in the transfer mechanisms (Thoma and Muth, 2016). In a recent review by Thoma and Muth (2016), the conjugative transfer in *Streptomyces* was shown to require a DNA-translocase, namely TraB. TraB resembles a protein involved in chromosome segregation during cell division, FtsK (Sepulveda et al., 2011).

Also, some plasmids or ICEs can be mobilized by other genetic elements. The mobilization may be due to the participation of proteins, which can act *in trans* (O'Brien et al., 2015; Ramsay et al., 2016; Torres Tejerizo et al., 2014), or through cointegration of the different elements (Brom et al., 2004; Haskett et al., 2016). The expression of transfer genes may be regulated through diverse mechanisms in different plasmids and ICEs. In this review, we survey different regulatory mechanisms present on plasmids and/or ICEs, aiming to discern if there is a correlation between the environmental conditions, the traits that are encoded in the mobile genetic elements, and their transfer regulation mechanisms.

2. Conjugative transfer of plasmids

Plasmids constitute part of the genomes of many different bacteria. Their sizes can vary from a few kilobases to about 2 megabases and can amount to 45% of the genome (Gonzalez et al., 2010; Romero and Brom, 2004; Thomas and Summers, 2008; Torres Tejerizo et al., 2011). Generally, they are thought to carry dispensable genetic information, useful only under very specific conditions, where they confer an advantage for maintenance of the population carrying them (Frost et al., 2005). However, some plasmids have been shown to carry essential genes (Landeta et al., 2011), allowing us to picture them as secondary chromosomes. The main feature distinguishing plasmids is that they replicate independently from the chromosome. They differ in the enzymes employed and in how the copy number is regulated (Thomas and Summers, 2008). Another relevant feature is the ability of plasmids to perform conjugative transfer, by themselves, when carrying all required elements (*oriT* site, Dtr and Mpf), sharing functions *in trans*, or through cointegration with other plasmids or ICEs, and they may also aid in the transfer of chromosomal markers (Frost et al., 2005). Plasmids contain genes required for their replication, and, many of them carry genes involved in conjugation (Frost et al., 2005; Thomas and Nielsen, 2005). In addition, they may carry an enormous variety of genes, conferring diverse phenotypic traits (Thomas and Summers, 2008). Since the 1960s, plasmids carrying antibiotic resistance genes have continuously gained interest (Wyrsch et al., 2016), moreover, pollution in different regions of the planet has led to the discovery and description of plasmids involved in metal resistance (Aminov, 2011). Plasmids involved in pathogenic (Aviv et al., 2016; Lang and Faure, 2014) and symbiotic interactions are also widely known (Romero and Brom, 2004). Similar to chromosomal genetic information, a high proportion of genes encoding proteins with unknown function is also found in

plasmids (Koonin, 2016). In order to better understand the mechanisms leading to bacterial diversification through conjugative transfer, we decided to study whether the observed modes of plasmid transfer regulation can be correlated to the traits encoded by the plasmids.

2.1. Symbiosis and pathogenicity plasmids

The genomes of rhizobial strains are usually composed of a high number of large plasmids, in addition to the chromosome. Some of these plasmids are involved in plant virulence, others in the establishment of nitrogen-fixing symbiosis with the roots of leguminous plants (symbiotic plasmids or pSyms), and others carry diverse genes involved in the utilization of carbon sources, transport systems, and unknown functions (Brom et al., 2000; Brom et al., 2014; Ding and Hynes, 2009; Lang and Faure, 2014). The transfer capacity of a number of these plasmids has been studied. In this section, we show that in many of them conjugation is regulated by different variations of quorum-sensing (QS). Briefly, QS is a regulatory mechanism that depends on the density of the population. The cells produce a signal molecule, usually a homoserine-lactone, which is excreted to the media and accumulates in a cell density-dependent manner. When the cellular population increases, the homoserine-lactone diffuses back into the cells and forms a complex with transcriptional regulators. This complex is able to induce different responses, such as the expression of the genes required for plasmid transfer (Nealson et al., 1970). The pTi plasmid from *Agrobacterium tumefaciens* was the first rhizobial plasmid described to regulate its conjugative transfer by QS. Plasmid pTi, so called for tumor induction, has two functionally distinct regions involved in transfer (Gelvin, 2000). The first one is related to the transfer of the T-DNA region to the host plant, through the action of the *vir* genes (for virulence). During the infection process, *A. tumefaciens* responds to phenolic compounds that are present at wound sites in the plant (Subramoni et al., 2014). *A. tumefaciens* is able to transfer a segment of the plasmid (T-DNA) to the host plant and integrate it into its chromosomal DNA, causing production of tumors (Kado, 2014). The T-DNA integrated in the plant's genome leads to the production of two classes of hormones: the first one corresponds to cytokinins and auxins that stimulate uncontrolled plant cell proliferation, allowing formation of the crown gall (Gohlke and Deeken, 2014). The second class comprises genes for the production of novel carbon compounds called opines. Opines can be used as carbon source by agrobacteria. Furthermore, they are required for induction of the second transfer system present in pTi, which allows transfer of the whole plasmid to other bacterial cells (Veluthambi et al., 1989). Opines control the expression of QS elements, which are required for the expression of the genes involved in conjugative transfer (Piper et al., 1993). This feature may be interpreted as that the utilization of specific carbon compounds contributes to the conjugative transfer of the plasmid. The whole system is very similar to the model LuxI-LuxR QS system described in *Vibrio fischeri* (Eberhard, 1972; Nealson et al., 1970). The main QS regulators in *A. tumefaciens* are encoded by pTi, including *traI*, *traR* and *traM* genes. TraI synthesizes a homoserine lactone, which binds to, and activates the TraR transcriptional regulator. TraR activates the transfer genes. *traM* encodes an antiactivator, which is able to bind to TraR when homoserine lactone levels are low, decreasing the conjugation levels (Lang and Faure, 2014).

In addition to pTi, the conjugative transfer of other rhizobial plasmids is also subject to regulation by QS, i.e.: the symbiotic plasmids of *Rhizobium leguminosarum* bv *viciae* 8401 (Danino et al., 2003; McAnulla et al., 2007), and of *Sinorhizobium fredii* NGR234 (He et al., 2003). Furthermore, non-symbiotic plasmids such as pRet42a of *Rhizobium etli* CFN42 (Brom et al., 2000; Tun-Garrido et al., 2003), pSfr64a from a bean-nodulating *S. fredii* GR64 strain (Cervantes et al., 2011) and pLPU83a from *Rhizobium favelukesii* LPU83, isolated from acid soils in Argentina, are also regulated by QS. pLPU83a shows functional versatility: it is able to use its own machinery, but also the transfer machinery encoded in other plasmids. Interestingly, although pLPU83a requires a *traR* gene to induce transfer in a QS-dependent manner, it lacks a homoserine lactone encoding gene, indicating the presence of a different regulatory network (Torres Tejerizo et al., 2014). In an indirect manner, transfer of the symbiotic plasmid of *R. etli* CFN42 is also subject to QS, because it may be transferred through cointegration with the QS-regulated plasmid pRet42a (Brom et al., 2004).

Another mechanism for conjugative transfer regulation that is present in various rhizobial plasmids depends on the RctA-RctB system. RctA is a repressor, structurally related to winged-helix DNA-binding transcriptional regulators. In pSym of *R. etli*, *rctA* is transcribed divergently from the *virB* operon, which encodes the Mpf system that participates in conjugation. RctA binds specifically to a site in the *virB* promoter, repressing its transcription. Additionally, the divergent localization of *rctA* and the *virB* operon generates transcriptional interference between them, modulating the expression of *rctA* (Pérez-Mendoza et al., 2005; Sepúlveda et al., 2008). Conjugative transfer is achieved in the presence of overexpressed *rctB,* because this regulator inhibits expression of *rctA,* allowing induction of the Dtr and Mpf genes. To date, an environmental signal allowing the induction of transfer of plasmids regulated by RctA-RctB has not been identified.

pSymA of *Ensifer meliloti* is also subject to regulation by RctA-RctB, however, the regulatory circuit presents some differences compared to the one operating in the *R. etli*

pSym. In the *E. meliloti* plasmid, *rctA* represses transfer genes, but the *rctB* ortholog activates transcription of conjugal genes without affecting *rctA* transcription. The expression of *rctB* in *E. meliloti* is controlled by *rctR* and *rctC*, which are absent in *R. etli*. While RctR represses *rctB*, *rctC* encodes a response regulator that binds to *rctR*, relieving transcription of *rctB* (Blanca-Ordóñez et al., 2010; Nogales et al., 2013). Unfortunately, the compound that activates *rctC* is still unknown.

Bioinformatic data have shown the presence of *rctA* and *rctB* homologues in the *A. tumefaciens* pAT plasmid, and recent transcriptomic data suggest that, at least in the pAT plasmid of strain P4, the regulation by RctA-RctB is under QS control (Mhedbi-Hajri et al., 2016; Nogales et al., 2013).

A different regulatory system has been described for pESI from *Salmonella enterica* sv infantis (Aviv et al., 2016). This plasmid is responsible for foodborne infections and confers multidrug resistance and virulence. Interestingly, transfer of this plasmid is modulated by conditions reminiscent of those present in the intestines of animals, such as temperature, oxidative stress and moderate osmolarity. The elements involved in the transcriptional regulation of pESI transfer are TraB and an oxygen homeostasis regulator, Fnr (Aviv et al., 2016).

2.2. Plasmids with antibiotic and metal resistance, and carbon compounds utilization

The discovery of antibiotics led to important medical advances in the fight against a number of bacterial diseases. However, it also led to an indiscriminate use of the antimicrobials. This has had a negative impact on the benefits of the use of antibiotics due to the rapid emergence of strains that have acquired resistance to the antibiotics. A "positive" aspect of this issue is that it has also allowed researchers to study how these resistance markers are distributed among the bacterial populations. Different studies propose that the presence of antimicrobial residues contaminating in groundwater, healthcare wastewater, etc., can provide a selective pressure for horizontal gene transfer (Baquero et al., 2013).

For instance, it has been shown that the combination of two antibiotics had a positive effect on the transmission of some conjugative plasmids that carry resistance genes to these antibiotics. Transfer of plasmids pRK2013, pSU2007, and RP4 from *Escherichia coli* DH5α to HB101 responds to low concentrations of kanamycin and streptomycin, which induce the transcription of two genes, *oppA* (oligopeptide-binding protein) and *rbsB* (ribose-binding protein), that participate in the conjugation process (Zhang et al., 2013), clearly evidencing the relation between the plasmid encoded genes and its conjugative regulation.

Biofilms are complex structures formed by microbial communities associated with different kinds of surfaces, and may facilitate conjugative transfer. In *Pseudomonas putida,* biofilm formation increases when bacteria are exposed to sublethal doses of different antibiotics (kanamycin, ciprofloxacin and norfloxacin), which, in turn, allows the transfer of plasmids carrying the different resistance cassettes. If this increase in conjugative transfer is solely due to the augmented population, or to a specific regulatory mechanism remains an open question, but also in this case, there seems to be a relation between the plasmid-encoded genes and transfer regulation. Furthermore, specificity in the effect is suggested by a new non-invasive method for the quantification of plasmid conjugation in biofilms. The use of fluorescent proteins, has shown an increase in conjugative transfer in biofilms exposed to sub-inhibitory concentrations of antibiotics for which some of the bacteria forming the biofilm are resistant, in contrast to the application of other antibiotics (Ma and Bryers, 2013). In recent work, Hu et al. (2016) made a comprehensive study of the effects of phylogeny and ecological distribution on the recent transfer of the mobile antibiotic resistance genes of 23,425 bacterial genomes. Their results showed a high proportion of the antibiotic resistance genes in Proteobacteria, and that these genes seem to transfer preferentially to phylogenetically close bacteria. The authors propose that the presence of antibiotics allows selection, while ecology and physical localization of the bacteria set limits to the conjugative transfer. How these items interact to achieve regulation of conjugative transfer is, at present unknown.

In a study directed to the evolution of plasmid host-range, Loftie-Eaton et al. (2016) showed that the transposition of Tn*6231* from the native mercury resistance plasmid pR28, encoding a putative toxin-antitoxin system, in combination with host mutations, significantly improved the persistence of antibiotic resistance plasmid pMS0506, in *Pseudomonas moraviensis*. This phenomenon shows evolutionary effects due to inter-plasmid events, and also may influence the spread of these mobile elements to other Beta and Gamma-Proteobacteria, when the resistance markers are moved to broad-host range plasmids, and consequently, they will be subject to different host-encoded regulators affecting their conjugative transfer.

Plasmids belonging to the IncA/C group are widely distributed among enterobacteria and have also been found in *Vibrio cholerae* strains. They have been shown to carry multidrug resistance elements, and are usually conjugative. Transfer of these plasmids is induced by AcaCD, whose expression is repressed by proteins Acr1 and Acr2. At present, the biological signal for relieving the repression and allowing AcaCD to induce transfer has not been identified (Carraro et al., 2016; Poulin-Laprade et al., 2015). AcaCD

has been labeled as "master regulator" because it modulates the expression of about two thirds of the plasmid genes (Carraro et al., 2015). Additionally, AcaCD has been shown to control the excision and transfer of a number of ICEs, linking the transfer of plasmids with that of genomic islands. (Carraro et al., 2015; Carraro et al., 2016; Poulin-Laprade et al., 2015).

Some Gram-positive bacteria use secreted peptides for intercellular communication. Dunny et al. (1978), reported the efficient conjugative transfer of a tetracycline resistance plasmid (pCF10) from *Enterococcus faecalis*. The mechanism leading to transfer is complex. It involves a signaling peptide (cCF10) encoded in the bacterial chromosome, which is ultimately responsible for induction of transfer, by allowing transcription of the *prg* operon. Conjugation only takes place in the presence of adequate recipients, this is ensured by the action of genes encoded in the plasmid, namely *prgQ,* whose product (iCF10) competitively inhibits cCF10, and by *prgY,* which reduces production of the peptide. In the recipients lacking pCF10, the peptide is synthesized and exported to the media. From there, it is transported into the donor by an oligopeptide permease. Both the inducer cCF10 and the inhibitor iCF10 are able to bind to the protein encoded by *prgX,* which is the regulator of the operon encoding transfer functions. The balance between cCF10 and iCF10 will determine whether the operon is induced or not. Additional control of the system is conferred by post-transcriptional modulation (Cook and Federle, 2014; Dunny, 2007; Dunny and Berntsson, 2016).

In another Gram-positive bacterium, *Clostridium perfringens*, toxin and antibiotic resistance genes are localized on conjugative plasmids closely related to pCW3, which has an atypical relaxase (Wisniewski and Rood, 2017; Wisniewski et al., 2016). The *tcp* region of pCW3 comprises 10 genes, and has been identified as the transfer region, showing some similarity to transfer genes of the conjugative transposon Tn*916* (Bannam et al., 2006). This region is present in all known conjugative plasmids of *C. perfringens.* The study of an avian necrotic enteritis strain of *C. perfringens* showed the presence of a plasmid that contains genes conferring bacitracin resistance. The *bcr* genes were localized on an ICE related to Tn*916* (Han et al., 2015). The identification of new genes involved in transfer, and of new combinations of phenotype conferring genes with ICEs, leads us to question how the distribution of these elements is regulated.

Regarding metals, different plasmids carrying genes conferring resistance have been described, for example, in *Cupriavidus metalliduran*s CH34, pMOL28 contains genes involved in resistance to Co(II), Cr(VI), Hg(II), and Ni(II); while pMOL30 confers resistance to Ag(I), Cd(II), Co(II), Cu(II), Hg(II), Pb(II), and Zn(II). These genes are localized in three putative genomic islands, one in pMOL28 and two in pMOL30. Transcriptomic data indicates that, among others, conjugative transfer proteins are upregulated (83 genes in pMOL28 and 143 genes in pMOL30) in the presence of high metal concentrations, suggesting that these conditions might be related to transfer of the plasmids (Monchy et al., 2007). A study of nonconjugative IncQ plasmids pMOL187 and pMOL222, encoding resistance to Cd, Zn, and Co and to Ni, Cd, and Co, respectively, demonstrated that persistence of the plasmids was dependent on cointroduction of the mobilizing RP4 plasmid in the microcosm, and that high metal concentrations, seemed to enhance transfer (Smets et al., 2003).

As early as 1993, it was shown that mercury released from dental amalgam fillings, contributed to the generation of strains carrying plasmids with multiple antibiotic resistance and mercury resistance genes (Summers et al., 1993). A more recent report of coexistence and possible dissemination of multidrug and metal resistant plasmids was published by Fang et al. (2016). In a very elegant manner, Klumper et al. (2017) have shown that the presence of low concentrations of metal stressors such as Zn, Cd, Cu, Ni and As decreased the transfer of a broad-host-range IncP-type plasmid (pKJK5) to potential recipients extracted from an agricultural soil, however, transconjugants were distributed among 12 different bacterial phyla, indicating that the distribution pattern was unaffected. Also, although the general tendency was to decrease transfer, for some specific OTUs transfer did actually increase. Overall, these results indicate a profound effect of metal-caused stress on conjugative transfer, measured as permissiveness, which has been defined as "the ability of a community to receive a plasmid, both in terms of transfer frequency and phylogeny" (Klumper et al., 2017).

Several mobile genetic elements have been described to have properties for utilization of carbon compounds (Don and Pemberton, 1981; Kinkle et al., 1993; Ohtsubo et al., 2012; Top et al., 1995). Plasmids pEMT1 and pEMT3 carry genes for degradation of 2,4-dichlorophenoxyacetic acid (Top et al., 1995). Top et al. (1998) showed that the presence of this compound increases the number of transconjugants in soil experiments.

3. Conjugative transfer of integrative and conjugative elements

ICEs are usually found integrated in the chromosomes of bacteria. Their replication is linked to chromosomal replication; nevertheless, some ICEs have *rep* proteins, and can replicate as circular elements after excision from the chromosome. Vertical inheritance is assured by the chromosome's replication and segregation. These elements are also able to conjugate, spreading genes horizontally. Details of

ICEs and excision mechanisms have been reviewed elsewhere (Johnson and Grossman, 2015; Wozniak and Waldor, 2010). ICEs are not restricted to specific taxa. Usually, they encode the whole machinery for the Dtr and Mpf, and thus, they are self-transmissible. For conjugative transfer, the ICEs must excise, re-circularize, transfer to the recipient cell, and there integrate into the "new" host genome. The regulation of the transfer of some ICEs has been described; in some cases, the traits encoded are involved in this regulation. Antimicrobials, stress-response and QS, among others, are indicated as regulators of gene expression of ICEs and their subsequent transfer. The molecular mechanisms of this phenomenon represent an interesting theme for relationships among bacteria in an ecological context, so, new cascades have been found for different elements. We aim to point out the general relationship among these traits.

3.1. Symbiosis and pathogenicity islands

Large ICEs are usually found in rhizobia, for example, ICE*Mls*sym^{R7A} was identified after a field experiment in which non-symbiotic rhizobia evolved to symbiotic rhizobia through the acquisition of a 500 Kbp genomic island (Sullivan et al., 1995; Sullivan and Ronson, 1998). This genomic island was described as ICE*Mls*sym^{R7A} (Ramsay et al., 2006). ICE*Mls*sym^{R7A} excision depends on an integrase called IntS and a factor that ensures recombination directionality, RdfS, while the integration of ICE*Mls*sym^{R7A} requires only IntS. Moreover, it was observed that the excision of ICE*Mls*sym^{R7A} changes depending on the growth phase of the bacteria, increasing at high cell densities (Ramsay et al., 2006). Later on, it was described that this event was QS-induced. Excision and transfer depends on the presence of *msi171* and *msi172*, whose expression relies on the promoter of *traI2* because all these genes are encoded in an operon *(traI2-msi171-msi172)*. As ICE*Mls*sym^{R7A} encoded two homoserine lactone synthase genes, *traI1* and *traI2*, and a LuxR-family transcriptional regulator, *traR*, QS regulation through homoserine lactones as in *Agrobacterium* pTi plasmid is present for the expression of *msi171* and *msi172* (Ramsay et al., 2009). Therefore, the transfer of nodulation genes carried by ICE*Mls*sym^{R7A} increases with high cellular density.

Recently, ICE^{Ac} was found in *Azorhizobium caulinodans*. This ICE^{Ac} of 87.6 Kbp harbors the nodulation genes needed to induce nodulation in *Sesbania rostrata*. Establishment of the nodulation process requires cross-talk between the plant and the bacteria, where the plant secretes flavonoids that induce the expression of nodulation genes in the bacteria (Jones et al., 2007). These nodulation genes are involved in the production of nodulation factors, which are small molecules that trigger several changes in the plant roots, preparing it for the infection. Surprisingly, flavonoids from *Sesbania rostrata* induce not only the expression of the nodulation genes present on the ICE^{Ac}, but also the conjugative transfer of this element (Ling et al., 2016).

The phytopathogen *Pectobacterium atrosepticum* SCRI1043 harbors 17 genomic islands. One of them, horizontally acquired island 2 (HAI2) has genes involved in pathogenesis (*cfa*-like polyketide phytotoxin clusters) (Bell et al., 2004). Excision and transfer of HAI2 is induced *in planta* by unknown compounds and is also modulated by QS (Vanga et al., 2015). In *Pseudomonas aeruginosa*, it was described that the self-transmissible pathogenicity island PAPI-1 carries over one hundred genes with several virulence features (Qiu et al., 2006). The transfer of this island required integrase gene *soj* and the *pil* operon encoding determinants of type IV pili (Carter et al., 2010; Qiu et al., 2006). Carter et al. (2010) observed that in stationary growth-phase the expression of the *pil* operon and *soj* is increased. This could mean that the transfer of PAPI-1 is also regulated by QS. Nevertheless, a more detailed analysis is needed.

3.2. ICEs with antibiotic or metal resistance, or carbon compounds utilization

As previously mentioned, several plasmids increase their transfer frequency when exposed to antimicrobials. In the case of some ICEs, the behavior is similar. Tn*916*, Tn*925* and CTnDOT are regulated by tetracycline. Tn*916* harbors a tetracycline resistance gene, when exposed to this antimicrobial, conjugative transfer increases 19-fold in *B. subtilis*; moreover, this frequency also increases when the element is transferred from other host strains (Showsh and Andrews, 1992). Ensuing studies showed that only the transfer frequency increases in the presences of tetracycline, while the excision frequency is not changed (Celli et al., 1997; Celli and Trieu-Cuot, 1998). Torres et al. (1991) demonstrated that Tn*925* also increased its transfer under tetracycline exposure. In *Bacteroides* sp., CTnDOT encodes for resistance to tetracycline and erythromycin (*tetQ* and *ermF*). When donor strains are grown in presence of tetracycline, regulatory genes *rteA*, *rteB* and *tetQ* are induced. *rteB* induces the transcription of *rteC*, which, in turn, activates the expression of the excision operon causing an increase in the excision frequency of the ICE, and thus, also in the transfer rate (Cheng et al., 2001; Johnson and Grossman, 2015; Sutanto et al., 2004; Whittle et al., 2001; Whittle et al., 2002; Wood and Gardner, 2015).

ICE*clc* from *Pseudomonas* sp. strain B13 carries genes involved in the degradation of carbon sources, such as 3-chlorobenzoic acid (Ravatn et al., 1998). As mentioned above, transfer of ICEs starts with the excision of the element; ICE*clc* integration and excision is catalyzed by the IntB13 integrase. The expression of this integrase is regu-

lated by growth conditions and environmental compounds, specifically, 3-chlorobenzoate. This reinforces the idea that the same compound that is able to induce the ICEs transfer, is also metabolized by the bacteria, through genes carried in the ICE (Johnson and Grossman, 2015; Sentchilo et al., 2003a; Sentchilo et al., 2003b).

3.3. ICEs with unknown functions

ICE*Bs1* from *Bacillus subtilis* does not carry symbiosis, pathogenicity or antibiotic resistance determinants. It is conjugative and is regulated by two modes. One of these is by DNA damage, involving the RecA protein; RecA promotes activity of ImmA, an anti-repressor which cleaves ImmR allowing excision and transfer (Auchtung et al., 2016; Bose and Grossman, 2011). The second mechanism relies on the cell density of potential donors. It involves protein the RapI, which is negatively regulated by the small peptide PhrI. Both, *rapI* and *phrI*, are encoded in ICE*Bs1*. PhrI is released to the media by the bacteria. When only a few bacteria harbor ICE*Bs1* (potential donors) but the total bacterial density is high (many potential recipients), the concentration of PhrI in the media is low, thus, it is not able to repress RapI and the transfer of ICE*Bs1* increases. When most of the bacteria harbor ICE*Bs1*, the concentration of PhrI in the media is high, hence RapI is repressed and the transfer of ICE*Bs1* is repressed. This mechanism prevents the transfer of the ICE to cells that already contain the element (Auchtung et al., 2016). A similar mechanism of attenuation of conjugative transfer was observed in *A. tumefaciens* (Cho et al., 2009), where entry exclusion prevents the acquisition of similar plasmids to the one already present in the strain. Another interesting feature of ICE*Bs1*, is its ability to promote transfer of some plasmids, previously considered to be non-mobilizable. The lack of a clear phenotype conferred by ICE*Bs1*, has led researchers to hypothesize that the advantage it provides to hosts carrying it is an increase in genome plasticity (Auchtung et al., 2016).

4. Other regulatory elements

High-throughput sequencing technologies and bioinformatic tools have allowed researchers to discover new genes, analyze genome arrangements and helped to understand the evolution of organisms. Nevertheless, this knowledge is restricted because of the high proportion of genes encoding hypothetical proteins, where no precise information related to their function is available. In recent years, several plasmid (and ICE) -encoded hypothetical genes have been shown to affect the regulation of conjugative transfer. The regulatory mechanisms have been thoroughly analyzed in some cases, however, the regulatory signals have still not been identified. In this section we survey some of them.

Ensifer meliloti LPU88 harbors pSmeLPU88a and pSmeLPU88b. pSmeLPU88b is able to transfer in the presence of pSmeLPU88a (Pistorio et al., 2003). *rptA* is a gene found in pSmeLPU88a, that regulates the transfer of pSmeLPU88b, but, the molecular mechanisms of its activation is not understood (Pistorio et al., 2013).

In *R. etli* CFN42 another modulator of conjugative transfer (RHE_PA00163) was recently described. This gene encodes a conserved hypothetical protein, which has orthologs distributed in a number of Proteobacteria. Mutations affecting this gene result in an increase in conjugative transfer of pRet42a (Lopez-Fuentes et al., 2015). Furthermore, Ding et al. (2013) and Lopez-Fuentes et al. (2015) described genes with XRE-domains, which regulate conjugative transfer. In *R. leguminosarum* bv. *viciae* strain VF39SM, pRleVF39b is regulated by *trbR*. A mutation in this gene leads to a 1000-fold increase in pRleVF39b transfer from a *Rhizobium* donor strain and a 100-fold increase from *Agrobacterium* donors (Ding et al., 2013). Also, gene RHE_PA00165 is harbored in pRet42a from *R. etli* CFN42. Mutations in this gene showed unaltered transfer frequencies when transferred from *R. etli*, but when *Agrobacterium* was used as donor, the frequency increased at least 10-fold (Lopez-Fuentes et al., 2015). Both genes have a helix-turn-helix XRE-domain. Furthermore, on ICE*Ml*Sym^{R7A} a gene (*qseC)* was found that also encodes a protein with an XRE domain. This protein modulates the excision and conjugative transfer of ICE*Ml*Sym^{R7A} through regulation of *qseM*, which is involved in the modulation of the master regulator, *traR* (Ramsay et al., 2013).

5. Host regulatory elements

Different stresses that instigate a SOS response in bacteria are also able to increase the transfer rates of ICEs. The SOS response can be induced by UV exposure, some antimicrobials (i.e. fluoroquinolones) and DNA damaging compounds, among others (Aminov, 2011). The induction of transfer in SXT-R391 and ICE*Bs1* occur through RecA-dependent excision (Auchtung et al., 2016; Johnson and Grossman, 2015; Wozniak and Waldor, 2010). In these examples, transfer of the ICEs is not modulated by an isolated trait, but by an integrated regulatory network, that assures the prevalence of the ICE in the environment.

Host-encoded elements also participate in the conjugation of diverse plasmids. Transfer of pSym of *R. leguminosarum* bv *viciae*, is induced by a chromosomally encoded homoserine lactone synthase, produced in the plasmid-lacking recipient, that is able to diffuse into the donor and activate the transfer system (Danino et al., 2003). In Gram-positive bacteria transfer is often induced by plasmid-less recipients, using chromosome-encoded factors

(Dunny and Berntsson, 2016; Goessweiner-Mohr et al., 2014). Transfer of *E. coli* plasmid F is influenced by the extracellular response element CpxA (Gubbins et al., 2002). In plasmid RA3 from the IncU group, the segregation proteins KorB and KorC repress the *mobC-nic* operon, which is required for DNA transfer (Kulinska et al., 2016; Ludwiczak et al., 2013). Also, both the virulence plasmid pSLT from *S. enterica* and the antibiotic resistance plasmid R100, originally isolated from *Shigella*, are conjugative in response to host-encoded regulators, such as Dam methylation and Lrp protein (Camacho and Casadesus, 2002; Camacho et al., 2005). Even in systems where a clear description of the participation of host-encoded factors is lacking, it is probable that this participation exists, and limits the transfer ability of plasmids and integrative elements.

6. Conclusions

In this review, we surveyed plasmids and ICEs encoding antibiotic and/or metal resistance, carbon compounds degradation, symbiosis or pathogenicity determinants. Although knowledge is still scarce regarding the environmental cues that trigger conjugation events, there are reports showing that specific antibiotics may induce conjugative transfer of some plasmids and ICEs. Many elements encoding features that involve interaction with other organisms, either beneficial (symbiotic) or harmful (pathogenic), are regulated by quorum sensing, a mechanism that is characterized by taking into account the impact of whole populations. Generally, the regulators are encoded in the transmissible structures. However, host features often participate in regulation of conjugative transfer by ICEs. This may be a consequence of their genomic localization. As they integrate into the host's chromosome, it can be speculated that ICE's temporarily form part of the cells background. This can also be applied to some plasmids, the classic example being *E. coli* plasmid F. Nevertheless, non-integrative plasmids also will affect bacterial fitness, and chromosomally encoded functions. Plasmids can be conjugative or mobilizable, helped *in trans* or through cointegration with other plasmids. The same applies to ICEs. Furthermore, there are plasmids that may help to mobilize ICEs, and vice versa, blurring the limits between these two kinds of elements. The examples presented here show the complexity of transfer regulation in many different plasmids/ICEs, generated by multiple layers of control.

Bacterial fitness can be defined as the relation between the capacity of the bacteria to use the environmental energy sources, its growth and the genes that are expressed during their life cycle. When a bacterial population colo-

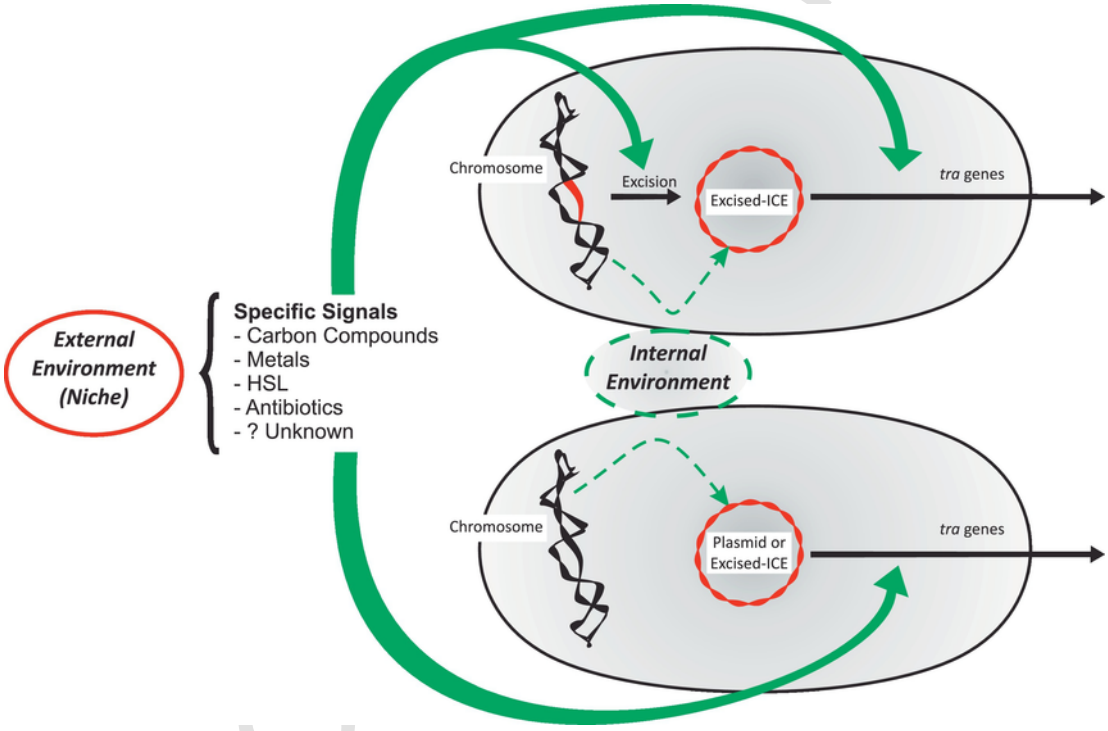

Fig. 1. Different levels modulate CT regulation. The upper drawing represents a cell containing an ICE inserted in the chromosome. The two points for regulation are excision and expression of transfer genes. The first level of regulation concerns the "internal environment" (interaction between the different genetic elements), the second level may be mediated by specific effectors related to the traits contained in the transmissible element, and the third level is the setting of limits by the external environment, due to the composition of the populations occupying the niche. The lower drawing represents a cell with a plasmid replicating autonomously or an excised-ICE. The levels of regulation are similar to those of the upper drawing, differing only in that there is no excision.

nizes a niche, the metabolism should be ameliorated to improve the colonization. In this context, it is logical that the genes needed for transfer are not expressed continuously, explaining their tight regulation.

The recent comprehensive analyses mentioned above point to a phylogenetic selection, implying that, above the specific regulatory elements, there is a higher regulation level, which depends on the "internal environment", defined as the interactions of the bacterial host's chromosome with the plasmids or ICEs, and the "external environment", corresponding to the habitat conditions and inhabitants. A scheme summarizing these ideas is presented in Fig. 1.

Currently, genomics constitute a huge source of information that allows the discovery of new genes, with unknown functions. Experimental work is needed to determine the function of these genes. In a kind of "back to the future" fashion, we should go back to the "wet" laboratory, to characterize new genes. In the context of this review, we propose that the unknown genes encoded in mobile genetic elements will be related to the conditions in the environment from which the elements were isolated, providing a cornerstone for the characterization of new molecules and mechanism of conjugative transfer regulation.

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