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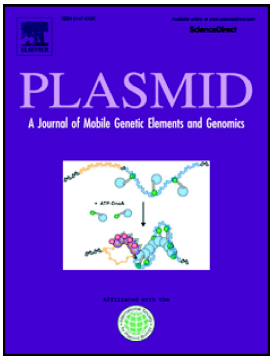


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Dolores Lucía Guzmán-Herrador, Matxalen Llosa

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# The Secret Life of Conjugative Relaxases

Dolores Lucía Guzmán-Herrador and Matxalen Llosa* llosam@unican.es

Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC), Universidad de Cantabria-CSIC-SODERCAN, Santander, Spain

*Corresponding author at: Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC)Universidad de Cantabria-CSIC-SODERCAN, Santander, Spain.

## Abstract

Conjugative relaxases are well-characterized proteins responsible for the site- and strand-specific endonucleolytic cleavage and strand transfer reactions taking place at the start and end of the conjugative DNA transfer process. Most of the relaxases characterized biochemically and structurally belong to the HUH family of endonucleases. However, an increasing number of new families of relaxases are revealing a variety of protein folds and catalytic alternatives to accomplish conjugative DNA processing. Relaxases show high specificity for their cognate target DNA sequences, but several recent reports underscore the importance of their activity on secondary targets, leading to widespread mobilization of plasmids containing an *oriT*-like sequence. Some relaxases perform other functions associated with their nicking and strand transfer ability, such as catalyzing site-specific recombination or initiation of plasmid replication. They perform these roles in the absence of conjugation, and the validation of these functions in several systems strongly suggest that they are not mere artifactual laboratory observations. Other unexpected roles recently assigned to relaxases include controlling plasmid copy number and promoting retrotransposition. Their capacity to mediate promiscuous mobilization and genetic reorganizations can be exploited for a number of imaginative biotechnological applications. Overall, there is increasing evidence that conjugative relaxases are not only key enzymes for horizontal gene transfer, but may have been adapted to perform other roles which contribute to prokaryotic genetic plasticity. Relaxed target specificity may be key to this versatility.


**Keywords:** Bacterial conjugation, Conjugative relaxase, Site-specific endonuclease, Genetic plasticity, Site-specific recombination, Rolling circle replication

## Introduction

Prokaryotes have successfully colonized the world thanks to their genetic plasticity. Horizontal gene transfer (HGT) is the main driver of this plasticity, and bacterial conjugation is one of the major HGT mechanisms, being responsible for the transfer of mobile genetic elements (MGE) and chromosomal DNA in both Gram-negative and positive bacteria. Evidences both from natural sources and experimental settings prove that conjugation can be a very promiscuous process, capable of mediating HGT between Gram-negative and positive bacteria, and even between prokaryotic and eukaryotic cells (1).

Bacterial conjugation is broadly defined as the transfer of DNA from one donor bacterium to one recipient bacteria which need to be in physical contact. This definition includes a set of processes with little in common, such as the Type VII-dependent transfer of chromosomal segments in mycobacteria (2), or the transfer of double-stranded DNA in a Type IV-independent manner in *Streptomyces* and other actinobacteria (3). In this review, we will refer only to conjugative transfer of single stranded DNA (ssDNA) through a Type IV secretion system (T4SS) in Gram-positive and –negative bacteria, which requires the action of a conjugative relaxase. Most of our knowledge has come from the study of conjugative and mobilizable plasmids, although in recent years it has become apparent that this mechanism is as frequent in plasmids as in Integrative and Conjugative Elements (ICEs), and both kind of elements share similar conjugative systems (4, 5). The conjugative DNA transfer process can be outlined as follows: in the donor cell, the DNA strand to be transferred is cleaved at the origin of transfer (*oriT*) by a site-specific endonuclease known as the relaxase,

which makes a covalent bond with the nicked strand; this nucleoprotein complex is transferred through a T4SS into the recipient cell, where the relaxase actively catalyzes the strand transfer reaction, leading to the end of the transfer process. This mechanism has been validated in different conjugative systems (6).

Conjugative relaxases are key enzymes in conjugative ssDNA transfer processes. They are characterized by their site- and strand-specific endonuclease activity. Initial characterization of relaxases from several different conjugative systems described them as proteins highly selective for their target DNA and which catalyzed transesterification reactions through a covalent adduct between the cut DNA and a catalytic Tyr residue. In support for this uniformity, the first solved crystal structures of several relaxases indicated that they all belonged to the HUH superfamily of site-specific single-stranded endonucleases. However, exceptions have become so numerous that the paradigm needs to be revisited. There are relaxases lying outside of the HUH superfamily; relaxases that do not use a catalytic Tyr; and relaxases which might not even make a covalent complex with the DNA. In particular, a growing number of recent reports show the ability of relaxases to act, with lower efficiency, on sequences other than their cognate targets, with intriguing biological consequences. The purpose of this review is to revisit the concept of conjugative relaxases, emphasizing the diversity rather than the unity, and questioning their target specificity to accomplish conjugative ssDNA as their only biological role.

## The growing family of conjugative relaxases

The name “relaxase” honors the pioneering work by Clewell and Helinski, who discovered the “relaxation complexes” formed by mobilizable plasmid ColE1, which, when isolated as a protein-DNA complex, underwent conversion from supercoiled to open circular form in the presence of denaturing agents (7). The authors soon discovered the strand specificity of the relaxation event (8). Discovery of the proteins responsible for this relaxation had to wait for almost two decades (9). Biochemical characterization of the covalent interaction between the relaxase and its cognate *nic* site was first reported for the TraI relaxase of IncP plasmid RP4 (10), and similar features were soon found for the relaxases of other conjugative and mobilizable plasmids (11-14). Relaxases were then related through a set of three conserved motifs to other ssDNA endonucleases involved in DNA replication and transposition (15, 16), which defined the HUH superfamily of site-specific ssDNA endonucleases. The HUH signature motifs were also found in relaxases from Gram-positive bacteria (17), leading to a proposal for a universal relaxase mode of action (18). Motif I contains the catalytic Tyr residue, which forms the covalent complex with the nicked DNA, while the HUH motif III , characterized by a set of three His residues, is important for coordination of the metal cation required for endonuclease activity.

There was an increasing need for relaxase classification, which led to several studies analyzing their taxonomy. **Table 1** summarizes current relaxase classification and their main biochemical and biological features. It is important to note that relaxases were phylogenetically analyzed according to their N-terminal 300 residues, which contain the catalytic domain; many relaxases harbor different C-terminal domains, which often play additional roles in the DNA transfer process. Known relaxases were grouped in six families by Garcillán-Barcia *et al* (19), although the

authors already proposed the existence of new families coming from uncharacterized transfer systems, where no relaxase homologue was apparent. The vast majority of relaxases possess conserved HUH motifs. This relationship among HUH relaxases

**Table 1.** Current classification and main features of conjugative relaxases (see text for details)

| **MOB Family** [1] | **F** | **P** | **Q** | **V** | **C** | **H** | **T** | **TcpM** [2] |
|---|---|---|---|---|---|---|---|---|
| **Prototype relaxase** | **R388-TrwC** | **RP4-TraI** | **RSF1010-MobA** | **pMV158-MobM** | **pAD1-TraX** | **GGI-TraI** | **Tn916-Orf20** | **pCW3-TcpM** |
| **3D Fold** [3] | **HUH** | HUH,HEN | **HUH** | **HUH** | **RE** | HD | Rep-trans | Y-rec |
| **Catalytic residue** | Tyr x2 | Tyr | Tyr | His | Tyr | Tyr | | Tyr |
| **Covalent complex** [4] | Yes | Yes | Yes | Yes | No | Yes? | | |
| **2nd Function** [5] | Pre Mob | Pre Mob rTn | Pre* Mob | Pre Mob Cop | Pre | Pre Rep | Rep | |

[1] As defined by Garcillán-Barcia *et al* (19) and Guglielmini et al (4).
[2] This relaxase was described after the MOB classification was reported, and does not fit into any of the defined families.
[3] Structural family based on the presence of signature motifs or 3D structure (in bold): HUH, HUH superfamily; HEN, HUH superfamily with variant HEN motifs; RE, restriction endonuclease; HD, HD hydrolase; Rep-trans, RCR initiation proteins; Y-rec, Tyrosine recombinase
[4] Yes, experimentally detected relaxase-DNA covalent complex. Yes?, indirect evidence suggesting protection of the 5`end of the T-DNA. No, searched but not detected. Blank, no information.
[5] Reported biological function other than conjugative self-transfer: Mob, in *trans* activity on heterologous oriT sequences; Pre, Plasmid Recombination Enzyme (Pre*, only on single-stranded substrates); Rep, initiator of plasmid replication; Cop, regulation of plasmid copy number; rTn, enhancer of retrotransposition.

would be confirmed by the resolution of the 3D structure of different members of the superfamily, which showed the conservation of the HUH catalytic fold (reviewed by Chandler *et al* (20)). Despite this conservation, some variants were reported: the

characteristic 3-His motif III was replaced by a HEN motif in relaxase MbeA of mobilizable plasmid ColE1 (21), and the third His is not conserved in a subset of $MOB_V$ relaxases (19). With respect to motif I, the $MOB_F$ family harbors several conserved Tyr residues, although the number and function of catalytic Tyr varies in each relaxase (22-24). A recent review summarizes the detailed knowledge that we have acquired on these canonical relaxases (25).

However, increasing knowledge of relaxases belonging to different families challenged this paradigm. Early works on relaxases of the $MOB_V$ family were unable to assign a catalyitic Tyr residue, in spite of their conservation of the HUH motifs (17, 26), and elucidation of the 3D structure revealed that these relaxases use a His residue instead of Tyr to make the nucleophilic attack and covalent complex (27). Another significant divergence was reported for the relaxase MobC of mobilizable plasmid CloDF13, the prototype of the $MOB_C$ family, which showed no homology to HUH relaxases; interestingly, the nicked *oriT* DNA did not have any blocked end, suggesting that covalent complexes were not formed (28). Modelling of the 3D structure of another relaxase of the $MOB_C$ family, TraX of plasmid pAD1 from *Enterococcus faecalis*, suggested a structure unrelated to the HUH fold, instead resembling restriction endonucleases. In spite of these structural differences, a Tyr residue was essential for the cleavage reaction, and a Tyr-mediated covalent adduct was proposed, although never detected (29). There are other relaxase families, less characterized, which do not include the HUH motifs. The best characterized examples are relaxase TraI of *Neisseria gonorrhoeae* GGI (30), representative of the $MOB_H$ family (19); Orf20 of conjugative transposon Tn*916* (31), representing family $MOB_T$ (4); and relaxase TcpM of the *Clostridium perfringens* conjugative plasmid pCW3 (32), which has not been assigned

to any MOB family. Although structural information is still lacking, these proteins do not resemble the previously characterized relaxases, and rather show similarity, or conservation of motifs, which relate them to HD hydrolases, Rep-trans proteins involved in RCR, and Tyr-Recombinases, respectively, highlighting the still underexplored diversity among conjugative relaxases. No covalent complexes have been reported for these divergent protein families, but it is not clear if this issue has been experimentally addressed. It must be taken into account that the covalent complex can be difficult to detect, as happened in the case of the filamentous phage fd, or the RepB replicase in plasmid pMV158, which required elaborated approaches to determine the existence of the covalent adduct (33, 34). The absence of a covalent complex with the relaxase would imply a substantial change in the current model for conjugative ssDNA transfer, which is based on the transfer of the nucleoprotein complex into the recipient cell, where the relaxase is required to terminate the transfer reaction. Surely, a deeper characterization of these novel families will determine if there is a covalent adduct, which requires a different methodology to be detected, or if ssDNA transfer by conjugation can be radically different in systems involving non-HUH relaxases.

Exploration of bacterial clades traditionally underrepresented has revealed new relaxase families, which await further study. Initial characterization of the relaxase RelLS20 from the *Bacillus subtilis* plasmid pLS20 showed the presence of HUH motifs and a catalytic Tyr residue, but no homology to previously defined relaxases. Interestingly, the authors found more than 800 genes in Firmicutes showing homology to this protein, which suggests RelLS20 is the prototype of a new family of relaxases restricted to this family of Gram-positives (35). Also, an extensive analysis of 124

genomes from 27 species of *Streptococcus* revealed 144 Integrative Mobilizable Elements, of which 118 harbored relaxases related to RCR Rep proteins, belonging to four totally new families, or to $MOB_T$ (36). In short, the diversity of relaxases has just begun to be revealed.

## Target specificity

Conjugative relaxases specifically bind to a target sequence in the *oriT*, and introduce a site-specific nick in the DNA strand to be transferred (*nic* site). The specificity of a relaxase for its target sequences was biochemically characterized initially for the $MOB_P$ relaxase TraI of the IncP plasmid RP4, using *in vitro* assays with labelled oligonucleotides (37). It was also determined that tight substrate binding and catalytic activity were independent (38). Similar experiments rendered equivalent results in the paradigmatic $MOB_F$ relaxases R388-TrwC and F-TraI (25). The elucidation of their 3D structures allowed fine mapping of the interactions with the DNA, leading to a detailed knowledge of the relevant protein residues as well as the *oriT* nucleotides important for the interaction. The relaxases bind to an inverted repeat near the *nic* site. The DNA requirements for specificity lie both in the DNA binding domain and in the cleaved site (25). The detailed structural and biochemical information showed that specificity relied on just a few protein-DNA interactions, thus suggesting that specificity might be altered by rational design. In fact, specificity swapping was obtained by changing only 4 bp of the *oriT*s of the staphylococcal mobilizable plasmids pC221 and pC223 (39), or two residues of the relaxases of plasmids F and R100 (40). Moreover,

González-Pérez *et al* (41) showed proof of principle that variant relaxases can be obtained that recognize the desired change in the target DNA.

Concerning the relaxases belonging to other families, the situation varies significantly. In the case of the $MOB_C$ relaxases, binding occurs specifically at a set of direct repeats located more than 70 bp away from the *nic* site (29). Two types of relaxases seem to be unable to introduce the site-specific nick by themselves. The $MOB_T$ relaxase Orf20 of Tn*916*, showed *in vitro* non-specific endonuclease activity, but sequence- and strand- specific cleavage was conferred by the Tyr recombinase responsible for integration / excision of the conjugative transposon (31). In the case of the TcpM relaxase of plasmid pCW3, which itself resembles Tyr recombinases, binding was specific for its *oriT* site, but DNA cleavage specificity could not be proven *in vitro,* suggesting other still unknown factors must confer specificity to this atypical relaxase (32). It is interesting to note that a set of $MOB_T$ relaxases recently described in streptococci have associated genes homologous to TcpA, the coupling protein associated with relaxase TcpM (36),which suggests that these two types of relaxases sharing non-specific endonuclease activity may share other evolutionary relationships on their respective transfer systems.

With few exceptions (42, 43), relaxases are shown to work in *trans* as efficiently as in *cis*. Thus, specificity can easily be checked *in vivo* by testing conjugal mobilization of DNA molecules containing different *oriTs*. Many reports confirmed that relaxases could mobilize plasmids containing their *oriT* site but not others, even if highly homologous. This was the case, for instance, for the related IncF plasmids F and R100 (40), the enterococcal plasmids pAD1 and pAM373 (44, 45), or mobilizable plasmids pC221 and pC223 (46). It is important in this context to distinguish between

binding/cleavage assays on oligonucleotides, and assays using supercoiled substrates with full *oriTs*. While the former address specifically the intrinsic binding/cleavage specificity of the relaxases, the latter mimic the *in vivo* process by including binding sites for accessory proteins, which are required to form the relaxosome, contributing to the extrusion of the binding site and exposure of the target as a single stranded region amenable to relaxase function (6). This role may also contribute in a decisive manner to plasmid specificity, such as in the case of the related IncP plasmids RP4 and R751, where the relaxases can be exchanged, but auxiliary factors could not, determining the *in vivo* specificity (47). Another example is the staphylococcal pWBG749 family of conjugative plasmids, where the SmpO accessory protein determines *oriT* specificity (48). In summary, most relaxases bind *in vitro* with high specificity to their target sequences, which is a prerequisite for conjugal transmission. *In vivo*, specificity involves a set of protein-protein and protein-DNA interactions among the relaxase, accessory protein/s, and the *oriT* site.

In spite of the specificity for their cognate targets, lower efficiency recognition of heterologous sequences has been reported for members of all families of HUH relaxases. For instance, the $MOB_F$ relaxases TraC of plasmids NAH7 and pWW0 could mobilize plasmids containing either *oriT*; in this case, the full *oriT* fragments shared only 63% identity, but the regions around the *nic* site were identical (49). Relaxase MobM from plasmid pMV158 was shown to relax *in vitro* other mobilizable plasmids from Gram-positive organisms, whose *oriT*s shared 67-100% homology with the pMV158 minimal *oriT* (50). Interestingly, not all relaxases are equally stringent on their DNA sequence requirements. The relaxases of the mobilizable plasmids pSC101 and R1162 (virtually identical to RSF1010), which recognize highly homologous *oriT*

sequences, nonetheless had different stringencies: while the relaxase of pSC101 could not mobilize RSF1010, MobA of RSF1010 could also act on the pSC101 *oriT* (51). The authors found that MobA could even initiate transfer from chromosomal sites, and discussed the implication of this promiscuity for horizontal gene transfer by this broad host range plasmid. A similar situation was reported in two other plasmids, which are totally unrelated except in their transfer regions: the enterococcal plasmid pCF10 and plasmid pRS01 from *Lactococcus lactis*. PcfG, the relaxase of plasmid pCF10, could mobilize plasmids containing the heterologous *oriT*, while the relaxase LtrB of pRS01 was specific (both *in vitro* and *in vivo*) for its own *oriT* (52). More surprisingly, the relaxase TrwC of plasmid R388 was shown to mobilize plasmids containing the *oriT* region of the Ptw plasmid of *Burkholderia cenocepacia*; while the relaxases of both plasmids are closely related, there is no significant homology among the *oriT* regions. The PtwC relaxase could not complement TrwC for mobilization of R388-*oriT* containing plasmids, although this could also be caused by a *cis*-acting preference (43).

The ability of some relaxases to cross-react on the *oriT* sequences targeted by other relaxases illustrates the biological relevance that their relaxed specificity may have for promiscuous horizontal gene transfer. This *trans*-mobilization phenomenon is more frequent than previously thought. Different strategies exist for achieving horizontal transfer by hitchhiking on the transfer machinery of co-resident plasmids (recently reviewed by Ramsay and Firth (53)). Mobilizable plasmids could be classified in the classical “ready-to-go” plasmids, which encode for their relaxase (and even for their own coupling protein, in the case of CloDF13 (28)), and “orphan” plasmids which rely solely on *oriT*-like sequences (sometimes encoding also for accessory proteins) to

be mobilized by the relaxases present in a co-resident plasmid. The latter are the outmost expression of this plasmid piracy, and represent the natural manifestation of a well-known laboratory fact: the *oriT* site is the only element of the conjugative machinery required in *cis*, and thus, any DNA molecule containing *oriT* can be mobilized if the appropiate transfer machinery is provided in *trans*. In staphylococci, a diverse range of such *oriT*-containing plasmids lacking any transfer gene, which have been associated with the spread of antibiotic resistance determinants, have been shown to be mobilizable by co-resident conjugative plasmids (48, 54). Another illustrative example of the power of this kind of low-cost mobilization can be found in the *Escherichia coli* plasmid pBuzz, less than 2kb in size, which relies on the conjugative machinery of a helper plasmid (55). These recent reports also searched for other potential *oriT*-containing plasmids and found many candidates, indicating that this is probably just the first glimpse of a widespread phenomenon.

In this new scenario, relaxases are not only responsible for the selfish transfer of the DNA molecule which encodes them, but also for *in trans* mobilization of opportunistic plasmids containing short sequences which resemble their targets. Harboring an *oriT*-like sequence could be a low-cost strategy for horizontal mobility, which relies on the presence of co-resident plasmids, but bypasses the added burden of maintaining dedicated transfer regions in their DNA. It is possible that many plasmids classified as non-mobile due to the absence of putative relaxases (56), may in fact be orphan mobilizable plasmids (53). *oriTs* alone can be more difficult to spot than when accompanied by relaxases or other conjugative functions. However, now that some reports have elaborated bioinformatics methods of detecting *oriTs* based on

sequence homologies and on structural features (57, 58), it can be anticipated that many more orphan mobilizable plasmids will be described.

## Moonlighting relaxases

Conjugative relaxases are classified as such based on their role in conjugative DNA transfer. Often, these enzymes are multi-domain proteins harboring other functional domains involved in the DNA transfer process. This is a frequent situation in the HUH relaxases, probably reflecting the modular evolution of this protein superfamily (20, 59). The covalently attached domains provide functions which either are essential or contribute to the efficiency of the conjugative transfer process, such as oligomerization, DNA binding, or the DNA helicase domain linked to the $MOB_F$ family of relaxases (25). Even the primase domain linked to the RSF1010 relaxase MobA, which is required for plasmid replication, was shown to increase the efficiency of conjugative DNA transfer, probably reflecting an adaptation of this broad host range plasmid to carry its own priming system to the recipient cell (60, 61). In many other occasions, however, relaxases behave as moonlighting proteins, performing additional functions independently of conjugation.

The ability of some conjugative relaxases to promote RecA-independent, site-specific recombination between two *oriT* copies was reported even before the characterization of these proteins as relaxases (62). *oriT*-specific recombination is dependent on the relaxase and occurs in the absence of the rest of the transfer machinery (63). Recombination can be intra- or inter-molecular, and relaxases can even catalyze the integration of the transferred DNA strand into a resident *oriT* copy in

the recipient (64). This site-specific recombinase/integrase ability has been reported for many relaxases, both from Gram-positive and –negative systems, belonging to different MOB families (reviewed by Wawrzyniak et al (65)), but it is not an inherent characteristic of relaxases; at this point it is unknown which factor(s) allow a relaxase to act as a site-specific recombinase. Probably, relaxases act only on single-stranded *oriT* copies, which can be generated by the action of accessory factors (66), or during the plasmid replication process, and completion of the reaction is mediated by the host-encoded replication/repair machinery (67). The *oriT* sequence itself also plays an important role, since the $MOB_H$ relaxase of ICE*clc* catalyzes recombination only on one of the two *oriTs* present in this ICE, while it can act on both *oriT1* and *oriT2* for conjugal DNA transfer (68). DNA sequence requirements at the different *oriT* copies involved in the recombination reaction suggested that recombination events mimicked the initiation and termination steps of conjugative DNA transfer (67, 69). In accordance with this idea, the target DNA requirements for integration of a relaxase-bound DNA strand are less stringent (70). In both conjugal DNA transfer and site-specific integration, tight controls restrict the initiation of the reaction, but once the covalent nucleoprotein complex is formed, the process can be finished with lower efficiency on DNA sequences differing from that of the cognate *oriT*. In this way, the cell ensures that the energy consumed to start the process will not be wasted vainly.

The biological function most obviously related to conjugative DNA transfer would be plasmid replication. Replication and conjugation are two faces of the same phenomenon: plasmid dissemination, either vertical or horizontal, respectively. In fact, early reports suggested that plasmids coordinate the decision-making process to decide whether to promote horizontal or vertical replication, depending on

environmental circumstances (71). The aforementioned primase domain linked to the conjugative relaxase MobA and involved in both plasmid replication and transfer would be another example of the close interrelationship between both processes. As already mentioned, most relaxases are evolutionarily related to RCR replicases: HUH Mob relaxases with HUH Rep proteins, and $MOB_T$ relaxases with Rep-trans proteins. In the last decade, different reports have highlighted the fact that both kind of proteins are functionally exchangeable to a certain extent (reviewed by Wawrzyniak et al (65)). Several HUH Rep proteins have been reported to initiate conjugal DNA transfer of their own replicons by cleaving the DNA at the nick *dso*, which then serves as an *oriT*. Conversely, ICE relaxases belonging to the $MOB_T$ and $MOB_H$ families were shown to initiate both conjugal transfer and vegetative replication of the ICE, which were considered, until then, unable to replicate autonomously.

A recent report constitutes an interesting addition to the catalogue of functions that conjugative relaxases can play, independent of conjugal DNA transfer. The relaxase MobM of the RCR plasmid pMV158 was found to participate in regulation of plasmid copy number by transcriptional repression of the antisense RNA, thus increasing the number of plasmid molecules ready to be transmitted, whether it is horizontally or vertically (72). Probably, the most unexpected function reported for a conjugative relaxase is the ability of LtrB, the relaxase of plasmid pRS01, to stimulate both the frequency and diversity of retrotransposition of a mobile group II intron, which resides precisely within the relaxase gene itself. LtrB was found to have weak off-target activity in addition to its *oriT*-specific cleavage activity; this introduction of spurious nicks would stimulate the frequency and density of intron mobility events (73). In this way, intron mobility is promoted when the conjugative relaxase is active,

i.e. during the conjugative process, thus stimulating the dissemination of the retrotransposon in donor and recipient cells.

## Biotechnological applications

The specificity of conjugative relaxases for their target sequences can be exploited for biotechnological purposes. The biological autonomy of promiscuous transfer systems provides an excellent source of basic building blocks for synthetic biology (74), and the use of relaxases and their target sequences for plasmid mobilization would be the most obvious example. The increasing collection of characterized relaxase/target DNA pairs allows for the generation of different plasmid combinations, which have been proposed also as computing wires in synthetic biological circuits for digital cell-to-cell communication (75). Relaxases can also be used for the sequence-specific modification of DNA-based nanostructures. Due to their covalent binding to specific single-stranded oligonucleotides, different target DNAs can serve as specific loading sites for their cognate relaxase. Proof of principle was obtained using the relaxases of plasmids R388, pKM101, RSF1010 and R100, and showing that each of them bound specifically to the oligonucleotide containing its target sequence, on two different types of DNA origami structures (76). The specificity of relaxases can be changed by rational design, as previously mentioned (41), and new substrates can be constructed by playing with the *oriT* elements which define binding specificity, rendering a wider catalogue of possible substrates to construct the nanostructures (77). Thus, relaxases constitute a potential new class of sequence-selective protein linkers for DNA nanotechnology, which can be used for the modification of DNA nanostructures *in vivo* and for biological generation of DNA–

protein hybrid nanostructures. In addition, relaxases are in general very permissive to fusions with other proteins of choice, maybe reflecting their own evolution (59), so they could be used as anchors for other relevant functional proteins.

The ability of some relaxases to catalyze site-specific recombination fits into many biotechnological applications, and it could be of special interest in microorganisms where there is a lack of genetic tools. A relaxase-based recombination system has been used in *Streptomyces coelicolor* to amplify gene clusters for antibiotic production, improving the yield (78). In another example, a site-specific recombination system was applied in *Bacillus* to obtain unmarked genetic manipulation by flanking the desired region with relaxase target sites (79). On the other hand, relaxed specificity could be useful in order to catalyze site-specific recombination or integration into a wide variety of DNA targets. As discussed above, the DNA specificity is very high at the start of the process, but less stringent on the second target to complete the reaction. This allows for strict choice of the DNA to be delivered, while having better options of finding the appropriate target in any given recipient genome (70).

As biotechnological tools, relaxases have the added bonus of being part of a horizontal DNA transfer system, and so they can be delivered *in vivo*, covalently linked to any DNA molecule of choice, into any cell capable of acting as a recipient in conjugation. This includes virtually any prokaryotic cell, and even eukaryotic cells (1). The use of T4SS targeting eukaryotic cells to deliver relaxase-DNA complexes into human cells has proven as an efficient alternative to conjugation (80, 81). Adding the appropriate secretion signal, different relaxases can be translocated through T4SS hosted by bacteria which target different human cell types (82).

The possibility of sending site-specific recombinases covalently linked to a foreign DNA molecule into specific human cells is a promising genetic tool (83). Attempts have been made to use relaxases for genomic modification in eukaryotic cells. However, the site specificity of the integration event is challenged by the overwhelming efficiency of so-called illegitimate recombination processes in the eukaryotic cell. Integration of DNA into the genome of plant cells is routinely accomplished using the conjugation-like system of the Ti plasmid of *Agrobacterium tumefaciens*, which has been the major tool for plant genomic modification for decades (84). A T-DNA strand covalently linked to the relaxase-like protein VirD2 reaches the nucleus thanks to the nuclear localization signals present in VirD2, and DNA is integrated in a non-specific manner. This integration process is mediated by the DNA polymerase theta (85), which promotes microhomology-mediated end joining. The fusion of a site-specific nuclease to VirD2 increased the specificity of the integration events in yeast cells (86). Conjugative relaxase TrwC was used to deliver DNA into human cells through the T4SS of bacterial pathogens. Analysis of integration events indicated that the vast majority of integration events were not sequence-specific, but interestingly, the integration rate was up to 100-fold higher than when foreign DNA was introduced by transfection or by another relaxase with no reported recombinase activity (87). TrwC-DNA complexes may account for this improvement in integration efficiency due to a protecting role of the DNA ends in the human cell, and/or the lack of specificity for the final target sequence to complete the site-specific integration reaction. This ability to promote integration could be combined with a site-specific endonuclease, as shown for VirD2, in order to accomplish *in vivo* delivery and site-specific integration of foreign DNA in the human genome.

## Biological implications

From a biological perspective, the high specificity of conjugative relaxases for their target sequences ensures that they transfer their own encoding DNA, as expected in a selfish DNA world. However, it becomes evident that relaxases are also involved in mobilization of other DNA molecules present in the same host, acting *in trans* on non-cognate targets. This phenomenon is probably much more widespread than currently thought, and it could happen that the contribution of relaxases to HGT is quantitatively higher by mobilizing orphan plasmids than its own replicon. Probably, these secondary targets have been evolutionary maintained as part of the many HGT strategies in prokaryotes.

The growing evidence of the ability of relaxases to perform functional roles independent of conjugative DNA transfer is also biologically significant. Their involvement in replication and recombination processes are not mere laboratory artifacts, since they have been validated in many instances, in unrelated systems, and with efficiencies well above biological noise. Relaxases acting as replication initiators highlight the common evolutionary origin and biological interplay between conjugation and replication (88, 89). The contribution of relaxases to the replication of an ICE is also a contribution to HGT, since this replication is essential to ensure that daughter cells inherit an excised form of the ICE. Site-specific recombination processes are important in plasmid evolution, creating replicons with mosaic structure and novel properties; the contribution of relaxase-mediated recombination events in plasmid evolution has been experimentally tested (90). A site-specific recombination event

involving a relaxase was found to be responsible for the amplification of an antibiotic-resistance determinant in *Enterococcus faecalis* (45). Other possible biological advantages of *oriT*-specific recombination events may be envisioned, such as dimer resolution, or formation of cointegrates to favor conduction by a helper plasmid. The ability to catalyze site-specific integration into target sequences present in the recipient genome constitutes an additional mechanism to mediate chromosomal integration of conjugative plasmids transferred into non-permissive hosts. The plasmids transfer range is usually broader than replication range (49), so a system facilitating integration in the chromosome will contribute to the colonization of new hosts, especially if the specificity for the integration target is more relaxed, as shown for the relaxase TrwC (70, 87).

**Figure 1** highlights the different biological functions attributed to conjugative relaxases. In summary, their secondary target, off-target and moonlighting activities all contribute in the end to increasing the genomic plasticity of prokaryotes, whether it is by directing horizontal transfer of self- or non-self DNA molecules, by contributing to plasmid stabilization through replication or increasing copy number, or by enhancing genetic rearrangements through recombination reactions, or promoting retro-transposition. Conjugative relaxases are considered as key contributors to the prokaryotic horizontal gene pool, but they may play other roles in prokaryotic evolution.

**Legend to FIGURE 1.** Schematic of functional diversity and biological relevance of relaxases. The arrows point to the different biological functions reported for conjugative relaxases. The thickness of the arrow is indicative of the dedication of relaxases to this function. Solid arrows represent functions based on specificity of the

relaxases for their target; dotted arrows represent functions derived from their activity on non-cognate targets or off-target. RLX, Relaxases; MOB, Mobilization; TRA, self-transfer; REP, Replication; COP, Copy number; REC, site-specific recombination; INT, site-specific integration; rTN, Retrotransposition; HGT, Horizontal gene transfer. The vertical arrow indicates the direction of the contribution of each layer to the following.

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## Highlights

- Analysis of conjugative relaxases from different bacterial clades is uncovering a diversity of structural folds and catalytic mechanisms
- Apart from conjugative self-transfer, relaxases mediate mobilization of other plasmids through activity on non-cognate *oriT*-like targets
- Moonlighting functions of conjugative relaxases include site-specific recombination and integration, initiation of replication, plasmid copy number control, or enhancement of retrotransposition
- Their relaxed specificity and moonlighting activity contribute to prokaryotic genetic plasticity and provide interesting biotechnological applications.