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Ordering the bestiary of genetic elements transmissible by conjugation

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Phylogenetic reconstruction of three highly conserved proteins involved in bacterial conjugation (relaxase, coupling protein and a type IV secretion system ATPase) allowed the classification of transmissible elements in relaxase MOB families and mating pair formation MPF groups. These evolutionary studies point to the existence of a limited number of module combinations in transmissible elements, preferentially associated with specific genetic or environmental backgrounds. A practical protocol based on the MOB classification was implemented to detect and assort transmissible plasmids and integrative elements from γ-Proteobacteria. It was called "Degenerate Primer MOB Typing" or DPMT. It resulted in a powerful technique that discovers not only backbones related to previously classified elements (typically by PCR-based replicon typing or PBRT), but also distant new members sharing a common evolutionary ancestor. The DPMT method, conjointly with PBRT, promises to be useful to gain information on plasmid backbones and helpful to investigate the dissemination routes of transmissible elements in microbial ecosystems.

Keywords: relaxase, plasmid classification, degenerate primer MOB typing, conjugation, ICE, transmissible elements

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Traditional Trends in Plasmid Classification

Plasmid biology emerged as a discipline with the study of fertility and resistance factors present in Enterobacteria, Pseudomonas and Staphylococci. At that emerging point, plasmids were assigned to different incompatibility groups (Inc), based on their ability to coexist in the same cell.[1] Incompatibility was generally (but not always!) an indicator of high similarity in the replication and/or partition modules of members included in the same Inc and of large differences among members from different Inc.[2,3] Many exceptions were found, as discussed previously.[4] Since replication is an essential plasmid property, Inc grouping continued to base plasmid classification in spite of the previous caveats. Subsequent experimental approaches moved to molecular biology techniques, such as DNA hybridization,[5] PCR (refs. 6–9, among others), and/or straight sequencing,[10,11] which provided direct information on the replicon DNA sequence.

Plasmid incompatibility testing was finally abandoned due to its technical drawbacks. Nevertheless, the tradition of grouping plasmids by Inc persisted, probably motivated by the fact that much of plasmid biology research during the following decades concentrated on a few plasmid backbones encoding virulence and antibiotic-resistance determinants, hosted in the "classical" bacterial families, mostly Enterobacteriaceae, where Inc testing was developed. Molecular approaches inevitably led to further Inc subdivisions, which split some Inc groups (IncQ,[12] IncH,[13] IncP-1,[14] IncF[15] and IncX plasmids[16]) and allowed the discovery of new ones (PromA,[17] IncR,[18] GR groups from *Acinetobacter baumannii*,[19] FII_K from *Klebsiella pneumoniae*, FII_Y from *Yersinia*, and FII_S from *Salmonella*[15]). For IncN, IncHI1 and IncI1 groups, a plasmid MultiLocus Sequence Tying (pMLST) approach was implemented to identify closely plasmid variants spread recently and assort them in numerous profiles (http://pubmlst.org/plasmid/). At the

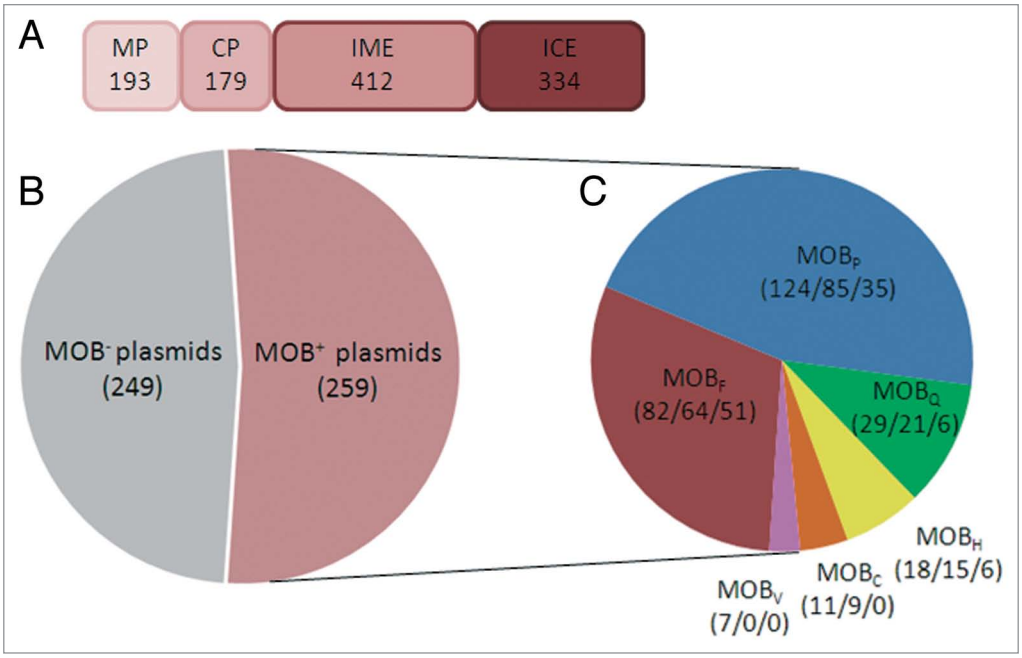

Figure 1. Bestiary of mobility elements. (**A**) Abundance of the different elements transmissible by conjugation. The number of conjugative plasmids (CP), mobilizable plasmids (MP), integrative and conjugative elements (ICE) and integrative and mobilizable elements (IME), identified in completely sequenced prokaryotes (1,207 chromosomes and 891 plasmids associated with chromosomes) is indicated for each element type and is proportional to each box width. Data were obtained from Dataset S1 in reference 33. (**B**) Proportion of transmissible (MOB^+) and non-transmissible (MOB^-) γ-Proteobacterial plasmids. (**C**) MOB family distribution in γ-Proteobacterial plasmids. For each MOB family which has representatives in γ-Proteobacteria the number of members is indicated by the first figure between parentheses. The second and third figures indicate the number of relaxases that can be detected by DPMT probes and the number of transmissible plasmids that can be detected by PBRT probes, respectively. Data presented in (**B and C**) were obtained from Supplemental Table 1 in reference 4.

end, the picture got even more inextricable and begged for the eclosion of a novel classification system.

Conjugation Modules and Types of Transmissible Elements

A classical plasmid backbone encompasses not only its replication module, but also partition and other maintenance systems as well as transfer functions. Transfer through conjugation is the main way used by plasmids for their dissemination among bacterial populations[20] and it is one of the strategies that contribute to their maintenance at a population level.[21,22] Hence, research attention focused on conjugation mainly due to its involvement in antibiotic resistance spreading. Details of the biochemical mechanism of conjugation and its ecological implications have been reviewed by references 23 and 24.

Different genetic modules encode the proteins involved in conjugation. The relaxase, sometimes helped by accessory proteins, recognizes a specific sequence of the transmissible element named origin of transfer (*oriT*), forming a relaxosome.[24] The relaxase cleaves one of the DNA strands at the *nic* site, resulting in a nucleoprotein complex that would be the translocated substrate.[24] The mating pair formation system (MPF) is the exit way of the conjugative substrate from donor to recipient cells[25] and an entry door for filamentous phages.[26,27] By linking the relaxosome to the transport channel, the coupling protein (T4CP) recognizes the substrate,[28-30] establishes contacts with the MPF[28,31] and pumps the substrate by its ATPase activity.[32]

Transmissible elements are classified according to their transfer ability in conjugative (those that code all components needed for transfer) and mobilizable (those that encode the MOB but not the MPF transfer functions and hence require a helper to be transferred). According to the genetic location of the transfer genes, conjugation-transmissible elements can be plasmids or integrative elements hosted in the chromosome. The mobile repertoire is thus composed of conjugative and mobilizable plasmids (CP and MP), integrative and conjugative elements (ICE), and integrative and mobilizable elements (IME) (**Fig. 1A**). The relaxase is the only common gene to all transmissible elements. Eight relaxase families (MOB families) were identified in a global survey of transmissible elements.[34] Phylogenetic relationships among relaxases constitute the rationale behind the MOB classification.[33,35,36] Based on the presence or absence of specific MPF genes and the phylogeny of the most conserved MPF protein (VirB4/TraU-like), seven MPF were detected.[33,34,37] Each MOB type is unequally distributed among plasmids of different sizes and different taxonomic classes. The association of a relaxase with a T4CP, as well as with each MPF type, is usually specific for each MOB family,[37] suggesting that the genes involved in conjugative transfer have evolved into specific sets co-adapted to specific physiological and ecological contexts. Thus, MOB types provide not only a classification tool

but also a valuable resource to predict the transfer characteristics of a plasmid and to follow its propagation routes in complex ecosystems.[38]

According to a genomic survey,[33] mobilizable elements outnumber conjugative elements both within integrated elements and within plasmids. Besides, the repertoire of ICE practically doubles that of CP (**Fig. 1A**). Furthermore, phylogenetic analyses indicate that CP and ICE show strikingly conserved patterns of conjugative genes. Exchanges of CP to and from ICE have been frequent along their evolutionary history. These findings suggest that CP often become ICE, and/or vice-versa, arguing for a unitary vision of the evolutionary dynamics of conjugative elements. For example, MOB_{H12} relaxases are encoded in both, conjugative plasmids (i.e., IncA/C) and ICE (i.e., IncJ elements).[4]

A Practical Approach to Classify Transmissible Elements According to their Relaxase: Pros and Cons

Most knowledge about conjugation comes from the study of plasmids and ICE hosted in Proteobacteria. The transfer systems of this phylum can be all grouped in six MOB and four MPF families.[37] In class γ-Proteobacteria, which includes a significant amount of genera involved in infectious diseases, five MOB relaxase families include more than 95% of the elements (MOB_F, MOB_P, MOB_Q, MOB_H and MOB_C) (**Fig. 1B and C**). A recently published screening method called "Degenerate Primer MOB Typing" (acronym DPMT) was developed to detect and classify relaxase genes carried by γ-Proteobacterial plasmids.[4]

Protein alignments of well-resolved clades in the five mentioned MOB phylogenies were analyzed to find blocks of residues with high global homology. Such blocks generally corresponded to catalytic motifs that contain sequence signatures of each MOB (sub)family.[35,36] Following the CODEHOP strategy,[39] degenerate primers, hybridizing to coding sequences of conserved amino acid motifs, were designed to amplify related relaxase genes. Such primers contained two regions: (1) a 3' core sequence (around 12-mer) that hybridized with the codons that determine the block of conserved amino acids and, therefore, was degenerate to encompass different codon usages; and (2) a 5' non-degenerate clamp sequence of variable length that contained a consensus of the most represented base at each position. The maximum degeneracy allowed in the oligonucleotide set was 24 (for a single primer) and 32 (for the sum of degeneracies of both oligonucleotides of a primer pair).

A set of 19 primer pairs was selected for its specificity and sensitivity using a collection of 33 reference relaxases. They represent more than 95% of the diversity of γ-Proteobacterial plasmids and are distributed in 16 MOB subfamilies with robust phylogenetic support. Once validated, DPMT was used to test two enterobacterial plasmid collections (originating from ESBL-resistant and pivmecillinam-resistant clinical isolates, respectively). In 93% of transconjugants, at least one MOB relaxase was detected by DPMT. The method detected not only relaxase genes identical to those already reported but also new MOB members ranging from 60 to 95% identity to the closer MOB homolog. These new branches, which populate known or new MOB subfamilies, will help to improve the MOB phylogenies and refine the DPMT set of primers. This fact underscores the power of DPMT to detect and classify plasmids that are undetected by other currently used methods (**Figs. 1 and 2**), singularly PBRT. Thus, DPMT is suitable for studying global plasmid diversity and finding deviant plasmids from well-studied backbones or those carried by a large number of taxonomic families. Other studies have also successfully used DPMT primer pairs conjointly with PBRT for identifying plasmids[4,40] and ICE[41] from clinical strains of Enterobacteria. ICE are gaining research momentum because of increasing evidence of their involvement in the dissemination of antibiotic resistance.[42] So, the ability of DPMT to detect them is valuable. The philosophy that guided the development of the γ-Proteobacteria MOB primer set has been extended to encompass relaxases of other taxonomical groups of bacteria, such as Enterococci.[43]

DPMT potency is achieved by a combination of phylogenetic support and the use of degenerate primers to uncover most codon variants of the relaxase signatures. As shown in **Figure 2**, DPMT finds and classifies backbones that share a common relaxase ancestor ("zoom out" strategy), while Inc classification and currently available PBRT and pMLST probes are useful at a lower phylogenetic depth, that is, at detecting practically identical backbones that carry different cargo genes ("zoom in" strategy). In a single MOB subfamily detected by DPMT, relaxases from different Inc sets can be grouped. On the other hand, plasmids of such Inc groups do not contain relaxases dispersed in different MOB subfamilies. Very few exceptions are observed to this statement, which can usually be explained by events of recombination, plasmid cointegration, and deletions of secondary replicons. In practical terms, only a few dozens of plasmid backbones are repeatedly detected in clinical and environmental isolates of γ-Proteobacteria, associated or not with antibiotic resistance genes.[23,44] Most of them are MOB^+. Therefore, a multiplex PCR MOB typing (MPMT) that uses a set of non-degenerate oligonucleotide primers is being presently developed to uncover the relaxases of those backbones, complementing PBRT in faster plasmid screenings (**Fig. 3**).

The relaxase-based plasmid classification was criticized by reference 45 with the argument that MOB and RepABC replicon phylogenies were not fully congruent. The main line of defense against that criticism is to mirror the problems of plasmid classification with those of bacterial classification. Even the concept of bacterial species is still controversial because of non-coherent phylogenies for some bacterial clades.[46,47] Nevertheless, new data emerged from metagenomic studies (instead of just 16S rRNA sequences) point to the existence of sequence-discrete populations, microbial communities predominantly organized in genetically and ecologically discernible populations, which possess the attributes expected for species.[48] Some degree of recombination between plasmids can be expected because more than one plasmid with homologous modules can coexist into the same cell.

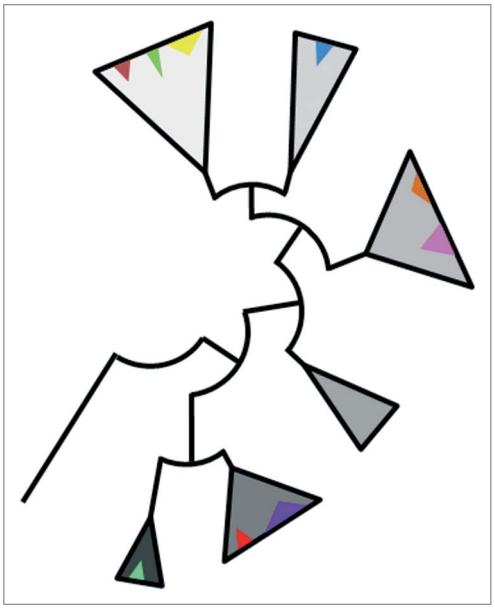

Figure 2. Phylogenetic depth of relaxase typing (DPMT) vs. Inc typing (PBRT). Schematic representation of an idealized relaxase family phylogeny. Grey-filled triangles represent monophyletic clades that constitute the different MOB subfamilies within a given family, and are thus detectable by a specific DPMT oligonucleotide primer pair. Smaller, colored triangles group relaxases belonging to a specific Inc plasmid group (which can be detected by a specific PBRT oligonucleotide primer pair). The width of the triangles is proportional to the number of relaxases they contain, while their height reflects the phylogenetic depth from the taxa to the last common ancestor of the group. Details on the correlation between Inc/REP types and MOB subfamilies for major plasmids of γ-Proteobacteria are provided in Figure 5 and Table 1 of reference 38, and Figure 8 and Supplemental Table S1 of reference 4.

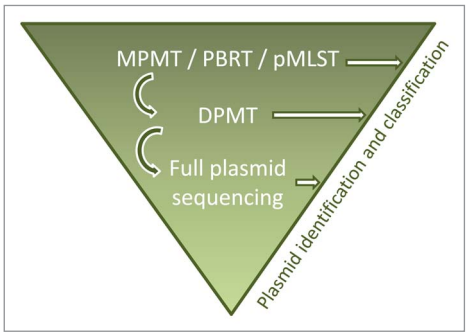

Figure 3. Workflow proposal for plasmid identification and classification. Plasmid DNA samples are first subjected to multiplex PCR MOB typing (MPMT) and/or PCR-based replicon typing (PBRT) and/or plasmid MultiLocus Sequence Typing (pMLST). Negative hits are subjected to DPMT-based MOB typing. If DPMT does not result in plasmid identification, full plasmid sequencing is performed. Positive hits (horizontal white arrows) represent plasmids that can be directly identified and classified. Negative hits (bent arrows), are subjected to the next classification protocol.

This is aggravated by the fact that plasmids are evolutionary units that propagate and replicate in different host structures, in turn influencing the selection of other units generated by introgressive descent.[49] By being part of the horizontal gene pool, plasmids explore many genomic landscapes where potential homologs can be found. The effect of recombination can be higher in the case of plasmids hosted in bacteria capable of natural competence for transformation, such as Firmicutes, α-, β- and ε-Proteobacteria.[50-53] Evidence suggests that there is not a single plasmid module that can escape from recombination. Even within the *repABC* family, horizontal gene transfer events of individual genes within the *rep* operon were detected. Besides, a particular phylogeny was found for each member of this operon, suggesting that each has its own evolutionary dynamics.[54] This fact does not necessarily invalidate a classification scheme, it only establishes the intervals of confidence in the inference of coevolution of different backbone modules.

While it is common to find plasmids with more than one replicon, plasmids with more than one relaxase gene are the exceptions, a fact that helps in the univocal classification of transmissible plasmids based on DPMT. On the other hand, only eight MOB families uncover the complete diversity of conjugative elements, while a higher and unknown amount of replication initiation protein families exists. There are even some plasmids (i.e., ColE1-like) that do not code for their own initiator protein. A tour de force was made 15 years ago by reference 55 to summarize the strategies the circular bacterial plasmids use to initiate replication and to control their copy number. Evolutionary studies of some replicon families have been already performed, such as: *repFIB*[56] and *repFIIA*[57] present in Enterobacteria, those related to RepA of plasmid R388,[58] *repABC* from α-Proteobacteria,[54,59,60] DnaA-like from Rhodobacterales[61] and 36 rep-families from Enterococci and Staphylococci.[8,9,62] Nevertheless, a phylogenetic analysis of the complete diversity of initiator proteins is still missing. Once achieved, such analysis, in combination with those performed for conjugative systems and other backbone regions such as the partition[63] and addiction systems,[64,65] would serve to evaluate the role played by recombination in the shaping of plasmid backbones in different phyla. Then, the common elements of the backbones of each family and the phylogenetic depth of stable assemblies of plasmid modules could be better analyzed. The sum of all these studies will hopefully provide a more accurate and operational classification based on a deeper knowledge of plasmid diversity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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