Chapter 3

Plasmid Replicon Typing

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Abstract

To facilitate the study of plasmids and their roles in human and animal health, environmental processes, and microbial adaptation and evolution, plasmid classification has been an important focus of plasmid biologists over the years. Initial schemes were based on the ability of a plasmid to inhibit F fertility, but due to certain limitations, these methods were superseded by incompatibility or Inc typing. Inc typing classifies plasmids by their ability to stably coexist with other plasmids in the same bacterial strain, a trait that is dependent on their replication machinery. Coresident plasmids are incompatible when they share the same replication mechanisms. Since plasmid replicon type determines Inc group, the terms *Inc* and *Rep* type to describe plasmid types are used interchangeably. Initially, Inc typing relied on introduction of a plasmid into a strain carrying another plasmid and determining whether both plasmids were stably maintained in the progeny. However, physical Inc typing is time consuming and not easily used in large-scale applications. Some of these shortcomings were addressed through development of a classification scheme based on identification of basic replicons using DNA hybridization and of a polymerase chain reaction (PCR)-based method of replicon typing enabling plasmid typing on a large scale. Here, we elaborate on a recently described PCR-based typing method that streamlines the typing of plasmids occurring among the Enterobacteriaceae; we believe the method will prove applicable to the study of plasmids on a large scale.

Key words: Inc typing, plasmid typing, plasmids, replicon typing.

1. Introduction

Plasmids are self-replicating, extrachromosomal units of DNA that encode nonessential but often valuable traits for their host bacterium *(1)*. Plasmids are a type of mobile genetic element (MGE), and as such, they are important agents of horizontal gene transfer (HGT). HGT of plasmids and other MGEs comprises an important source of genetic information for their bacterial

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hosts. In an avian pathogenic *Escherichia coli* (APEC) strain from which the genome was recently sequenced, it was shown that about 10% of the total genetic information was contained within four plasmids *(2)*. The traits encoded by these four plasmids included virulence factors, fitness factors, and genes encoding resistance toward antibiotics, disinfectants, and heavy metals *(3,4)*. In addition, these plasmids contained genes encoding for their dissemination and stability *(3,4)*. In general, such a pool of mobile genes (the mobilome) likely plays a crucial role in microbial evolution, providing bacteria a means to compensate for their lack of sexual reproduction, the major mechanism of genetic innovation in higher organisms *(5)*. Acquisition of such "ready-made" genes on plasmids and other MGEs enables the host bacterium to respond quickly to environmental changes, such as introduction of disinfectants and antibiotics. This would not be the case if bacterial fitness were solely reliant on *de novo* evolution *(5)*.

Plasmids contain genes necessary for initiation and control of replication and include accessory genes that encode a wide variety of phenotypes that help their bacterial hosts exploit and adapt to their environments *(6,7)*. These traits are considered accessory functions and include antibiotic and heavy metal resistance, metabolic properties, and pathogenicity. Such phenotypes have important implications for human and animal health, environmental processes, and microbial adaptation and evolution. In recognition of the importance of plasmids in these processes and to facilitate their study, plasmid classification has been an important focus of plasmid biologists over the years. Initial schemes were based on the ability of a plasmid to inhibit F fertility *(8)*, but due to certain limitations, these methods were superseded by incompatibility or Inc typing in the 1970s *(8)*. *Incompatibility* typing classifies plasmids by their ability to stably coexist with other plasmids in the same bacterial strain. Incompatibility is defined as the inability of two plasmids to be stably inherited in the absence of external selection *(1)*. Plasmids that are incompatible with one another are assigned to the same incompatibility or Inc group, while those that can exist together generally belong to different incompatibility groups. Coresident plasmids are defined as incompatible when they share the same replication mechanisms. Since plasmid replicon type determines Inc group, the terms *Inc* and *Rep* type to describe plasmid types are used interchangeably *(1)*.

Initially, Inc typing relied on introduction of a plasmid into a strain carrying another plasmid and determining whether both plasmids were stably maintained in the progeny *(1)*. Unfortunately, physical Inc typing proved a time-consuming task that was fraught with shortcomings. Couturier *et al.* addressed some of these shortcomings through development of a classification scheme based on identification of basic replicons using DNA:DNA hybridization *(9)*. Sobecky *et al.* showed that this method could

be applied to identify plasmid replicon types among isolates from complex marine microbial communities ***(10)***. Similarly, Mainil *et al.* demonstrated that this technique could be used to identify replicon types among the virulence plasmids of enterotoxigenic *E. coli* ***(11)***. However, the use of this technique was still extremely laborious, making typing of plasmids in large bacterial populations problematic. The advent of the polymerase chain reaction (PCR) made new time-saving methods of plasmid typing possible. Carattoli *et al.*'s PCR-based method of replicon typing enabled plasmid typing on a large scale ***(12)***. This method employs five multiplex and three simplex PCRs to recognize the major plasmid Inc groups occurring among the Enterobacteriaceae. The utility of this method for epidemiological studies was demonstrated with study of the plasmids encoding resistance to the newer β-lactam antibiotics among *Salmonella* and *E. coli* ***(13,14)***. Recently, we have simplified this replicon typing scheme to efficiently detect the presence of 18 replicon types occurring among the Enterobacteriaceae ***(15)***.

The original procedure used by Carattoli *et al.* involved five different multiplex panels each recognizing three replicon types and three simplex PCR reactions for the F, K, and B/O replicon types ***(14)***. We have made modifications to this procedure to make it more cost-effective and faster for screening large bacterial populations. First, instead of using a genomic purification kit to prepare template DNA, we used boiled lysates as a source of template DNA, as described by Johnson and Stell ***(16)***. This technique is a fast, inexpensive method for producing total DNA template suitable for PCR. The second modification was to reduce the total number of PCR panels used in the assay. **Table 1** lists the primers used within each panel, the target of each primer pair, and the expected amplicon sizes. This protocol screens for 17 gene products.

2. Materials

2.1. Cell Culture and Control Strains

1. MacConkey's agar plates (BD Diagnostic Systems).
2. Luria broth (LB) (BD Diagnostic Systems).
3. Control strains: The original control strains used for this procedure are available on request from Alessandra Carattoli, Istituto Superiore di Sanità, Rome, Italy ***(14)***. These controls were created by cloning the PCR product of each replicon type amplified into the pULB plasmid vector. The targets for each plasmid replicon include replication genes, *ori* sites, iteron sequences, and plasmid-partitioning genes specific for

Table 1
Primers and Controls Used in This Procedure

Panel	Inc type	Target	Amplicon size		Primer sequence
1	B/O	RNAI	159	F R	GCGGTCCGGAAAGCCAGAAAAC TCTGCGTTCCGCCAAGTTCGA
1	FIC	*repA2*	262	F R	GTGAACTGGCAGATGAGGAAGG TTCTCCTCGTCGCCAAACTAGAT
1	A/C	*repA*	465	F R	GAGAACCAAAGACAAAGACCTGGA ACGACAAACCTGAATTGCCTCCTT
1	P-1 alpha	Iterons	534	F R	CTATGGCCCTGCAAACGCGCCAGAAA TCACGCGCCAGGGCGCAGCC
1	T	*repA*	750	F R	TTGGCCTGTTTGTGCCTAAACCAT CGTTGATTACACTTAGCTTTGGAC
2	K/B	RNAI	160	F R	GCGGTCCGGAAAGCCAGAAAAC TCTTTCACGAGCCCGCCAAA
2	W	*repA*	242	F R	CCTAAGAACAACAAAGCCCCCG GGTGCGCGGCATAGAACCGT
2	FIIA	*repA*	270	F R	CTGTCGTAAGCTGATGGC CTCTGCCACAAACTTCAGC
2	FIA	Iterons	462	F R	CCATGCTGGTTCTAGAGAAGGTG GTATATCCTTACTGGCTTCCGCAG
2	FIB	*repA*	702	F R	GGAGTTCTGACACACGATTTTCTG CTCCCGTCGCTTCAGGGCATT
2	Y	*repA*	765	F R	AATTCAAACAACACTGTGCAGCCTG GCGAGAATGGACGATTACAAAACTTT
3	I1	RNAI	139	F R	CGAAAGCCGGACGGCAGAA TCGTCGTTCCGCCAAGTTCGT
3	F	RNAI/*repA*	270	F R	TGATCGTTTAAGGAATTTTG GAAGATCAGTCACACCATCC
3	X	*ori*	376	F R	AACCTTAGAGGCTATTTAAGTTGCTGAT TGAGAGTCAATTTTTATCTCATGTTTTAGC
3	HI1	*parA-parB*	471	F R	GGAGCGATGGATTACTTCAGTAC TGCCGTTTCACCTCGTGAGTA
3	N	*repA*	559	F R	GTCTAACGAGCTTACCGAAG GTTTCAACTCTGCCAAGTTC
3	HI2	Iterons	644	F R	TTTCTCCTGAGTCACCTGTTAACAC GGCTCACTACCGTTGTCATCCT
3	L/M	*repABC*	785	F R	GGATGAAAACTATCAGCATCTGAAG CTGCAGGGGCGATTCTTTAGG

See ding IncP primers.

that particular replicon (**Table 1**). In our protocol, we also use a set of *E. coli* control strains that harbors wild-type plasmids in their natural state. Because most of these plasmids occur in low copy, we have found that stronger products are often observed for the cloned pULB controls as compared to the wild-type controls. Therefore, we include wild-type controls to ensure that our sensitivity is high enough to detect naturally occurring low-copy plasmids. We make these wild-type controls available on request.

4. 1.5-mL centrifuge tubes (Fisher Scientific).

2.2. Polymerase Chain Reaction

1. Amplitaq Gold Taq polymerase (Applied Biosystems).
2. dNTP (deoxynucleotide 5′-triphosphate) mix (USB Corp.). The dNTP mix, purchased at a concentration of 10 m*M*, is diluted 1:4 in nanopure water to give a working concentration of 2.5 m*M* and is aliquoted into 1.5-mL microcentrifuge tubes in volumes of no more than 400 μL to avoid repetitive freeze-thaws.
3. Primers (IDT Technologies, Coralville, IA): Oligonucleotides are purchased at the 25 nmol amount with standard desalt. When received, the primers are resuspended to a concentration of 0.1 m*M*. For each PCR panel (*see* **Table 1**), equal amounts of each primer are combined into a pooled primer tube. For example, we typically combine 50 μL of each resuspended primer in the panel.
4. PCR buffer (Applied Biosystems).
5. $MgCl_2$ (Fisher Scientific).
6. PCR tubes (Fisher Scientific).
   Amplitaq Gold Taq, dNTP mix, IDT Technologies primers, PCR buffer are listed because we wish to provide exact details regarding the optimization of this protocol. This is not an endorsement for these products. Other products could be substituted in their place but may require further optimization of the procedure.

2.3. Agarose Gel Electrophoresis of PCR Products

1. SeaKem Agarose (Lonza Bioscience).
2. Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (Fisher Scientific).
3. Loading buffer (40% glycerol, 0.4% bromophenol blue in distilled water).
4. Ethidium bromide (Sigma Aldrich Corp.).
5. DNA molecular weight marker (Minnesota Molecular Inc.).

3. Methods

3.1. Day 1

Streak out the pULB control strains, the wild-type strains, and any experimental strains to be tested on MacConkey's agar. Incubate overnight at 37°C.

3.2. Day 2

1. Prepare 1.5-mL centrifuge tubes for each strain containing 1 mL of LB broth.
2. Autoclave the tubes and allow them to cool to room temperature.
3. Inoculate a single colony of each streaked strain into each of the tubes.
4. Incubate overnight with moderate shaking at 37 °C.

3.3. Day 3

Prepare template DNA from the LB cultures using the boiled lysate method *(16)*.

3.3.1. Preparation of Template DNA

1. Preheat a dry heat block to 100 °C.
2. Centrifuge the LB cultures at 12,000*g* for 1 min.
3. Pour off the supernatant and blot the tubes on a dry paper towel.
4. Resuspend the bacterial pellets in 200 µL of nanopure water.
5. Incubate the tubes at 100°C for 10 min.
6. Centrifuge the tubes at 12,000*g* for 1 min.
7. Withdraw the supernatant from each tube and place in a clean 1.5-mL microcentrifuge tube. This is your template DNA to be used in the PCR reaction.

3.3.2. Polymerase Chain Reaction

1. Thaw boiled lysate template, 10X PCR reaction buffer, 2.5 m*M* dNTP mix, primer pools, and nanopure water on ice.
2. Add 2 µL of each boiled lysate template to appropriate 0.2-mL PCR tube.
3. Assemble master mix as follows (volumes are given per reaction):
   2.5 µL 10X PCR buffer
   2.0 µL dNTP mix
   4.0 µL $MgCl_2$
   1.0 µL primer pool
   0.25 µL Taq polymerase
   13.25 µL nanopure water
4. Mix well and add 23 µL of master mix to each reaction tube (hot start not necessary; *see* **Note 1**).
5. Subject tubes to the following cycling conditions in a thermal cycler:
   Step 1. 94°C for 5 min

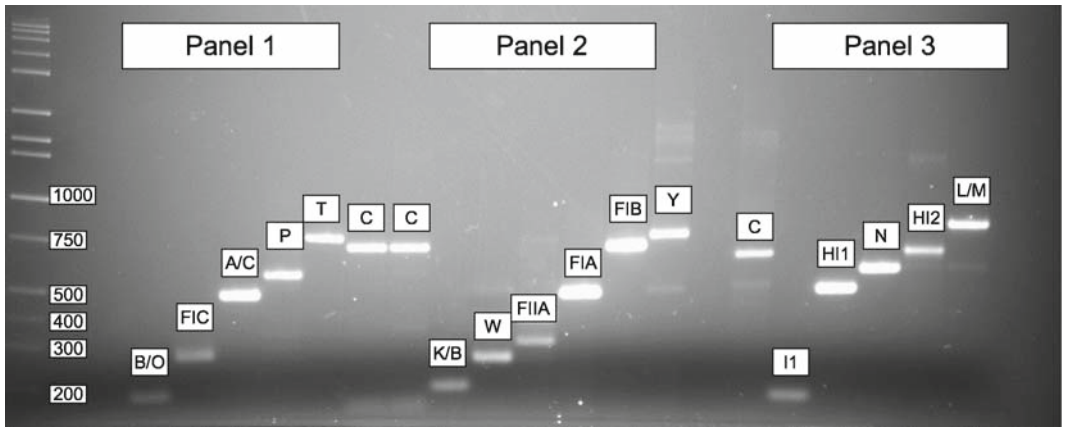

Fig. 1. Agarose gel electrophoresis of control strains for plasmid replicon typing procedure. *C* wild-type control strains. First C depicts *E. coli* strain APEC O1, harboring the FIB replicon in panel 2. Second C depicts *E. coli* strain APEC O2, also harboring the FIB replicon. Third C again depicts APEC O1, harboring the HI2 replicon in panel 3. Note that the F and X amplicons are not included in this image. The use of these primers is optional. The X replicon is extremely rare among Enterobacteriaceae, and the F replicon is an additional option for further confirmation of the presence of an F-type plasmid.

Step 2. 94°C for 30 s

Step 3. 60°C for 30 s

Step 4. 72°C for 90 s

Step 5. Steps 2–4 are repeated 29 times

Step 6. 72 °C for 5 min

3.3.3. Electrophoresis

1. Run 12 μL of each reaction on a 1.5% (w/v) agarose gel to visualize products.
2. Interpret the results by comparing the known controls (**Fig. 1**) to the experimental strains (*see* **Notes 2** and **3** regarding assay specificity and multiple products). It is good to run a linear DNA marker that has bands ranging from at least 50 to 1,000 bp. There should be at least one band per 100 bp on the low end of the marker.

4. Notes

1. In our experience, a hot start PCR is not necessary for this procedure. With our reagents on ice, we assembled the PCR reaction at room temperature without any adverse effects. This procedure was optimized using AmpliTaq Gold DNA polymerase. While other Taq polymerases could be used, their use may require some optimization.

2. This protocol was designed to err on the side of caution *(14,15)*. That is, the primers used here are more likely to miss a product than they are to detect a false-positive reaction. Because plasmids have a high degree of plasticity, this assay is not perfect. Compounding the complexities of plasmid plasticity is the general shortage of whole-plasmid sequences available for analysis. Very recently, several plasmid genome-sequencing projects have increased the number of replicon sequences available for comparative analyses (http://ecoli.cvm.iastate.edu and http://www.sanger.ac.uk). A comparison of the primer sequences in this protocol with those newly available plasmid sequences revealed that the protocol accurately detects most of the sequenced plasmids from each known incompatibility group. However, it is also evident from these analyses that some plasmids may not be detected. Future efforts will need to focus on the further development of this technique to all plasmid variants. At present, though, this protocol is a fast, inexpensive method for determination of most of the plasmid replicon types occurring among large populations of members of Enterobacteriaceae.
3. In our experience, double bands or bands of unexpected size can sometimes occur with the FIIA primers. Also, faint high molecular weight bands are sometimes observed inexplicably. However, these bands do not appear to have an effect on the assay as they have been observed in our sequenced wild-type controls and have not affected the expected products. As mentioned, some replicon types are highly variable. As a result, an occasional false negative could occur due to this variability. The replicons that have a high degree of variability include FIIA, L/M, FIA, and FIC.
4. IncP plasmid replicon: It is important to note that the primers in this procedure only detect the IncP-1 α plasmid replicon type. While the multiplex panels do not detect the IncP-1 β replicon type, other PCR-based protocols exist for its detection *(17)*.

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