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Iteron control of *oriV* function in IncP-1 plasmid RK2

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ABSTRACT

Replication control of many plasmids is mediated by the balance between the positive and negative effects of Rep protein binding repeated sequences (iterons) associated with the replication origin, *oriV*. Negative control is thought to be mediated by dimeric Rep protein linking iterons in a process termed "handcuffing". The well-studied *oriV* region of RK2 contains 9 iterons arranged as a singleton (iteron 1), a group of 3 (iterons 2–4) and a group of 5 (iterons 5–9), but only iterons 5 to 9 are essential for replication. An additional iteron (iteron 10), oriented in the opposite direction, is also involved and reduces copy-number nearly two-fold. Since iterons 1 and 10 share an identical upstream hexamer (5' TTTCAT 3') it has been hypothesised that they form a TrfA-mediated loop facilitated by their inverted orientation. Here we report that contrary to the hypothesis, flipping one or other so they are in direct orientation results in marginally lower rather than higher copy-number. In addition, following mutagenesis of the hexamer upstream of iteron 10, we report that the Logo for the hexamer "upstream" of the regulatory iterons (1 to 4 and 10) differs from that of the essential iterons, suggesting functional differences in their interaction with TrfA.

1. Introduction

Resistance to antibiotics is one of the most important healthcare issues currently facing mankind and many different creative approaches are needed to find solutions (Church and McKillip, 2021; Czaplewski et al., 2016). Since many antibiotic resistance genes are plasmid-borne and numerous plasmids carry multiple resistance genes, displacing such plasmids from bacterial populations may provide one line of approach to prolong the usefulness of existing antibiotics (Kamruzzaman et al., 2017). Using the complex F-like plasmids, that are prevalent in *Enterobacteriaceae* as a model target, we have developed an effective plasmid "curing" (displacement) strategy by blocking replication and neutralising addiction systems of the target plasmid (Hale et al., 2010). In exploring how this can be turned into a therapy we have tested IncP-1α plasmid RK2 (60,096 bp) as a vector because of its broad host range (BHR), which may facilitate its spread through complex bacterial communities like the gut microbiota (Tannock, 2022). We found that RK2 itself was not very effective as a vehicle, giving only low curing rates, but that deletions removing parts of the replication origin involved in plasmid copy-number control could make it much more effective

(Lazdins et al., 2020). We have therefore re-examined the control elements in the replication origin with the aim of understanding how plasmid curing can be potentiated.

RK2 has a low copy-number (around 4–8 copies per chromosome in *Escherichia coli*) and is stably maintained in diverse Gram negative bacterial species (Thomas and Smith, 1987). It is a conjugative plasmid containing *oriT* in its *tra* region and an additional *trb* region encoding a Type IV Secretion system (T4SS) that produces the essential pilus (Pansegrau et al., 1994). The other essential backbone functions are a vegetative replication origin (*oriV*) and a gene encoding a replication initiation protein (*trfA*), which is regulated by the products of a dual partitioning and central control region (ccr) encoding *korA, incC* and *korB* (Kolatka et al., 2010). The broad-host-range and self-transmissibility make RK2 a suitable vector for genetic manipulations and a very good model to study plasmids in bacteria. Examination of RK2 replication intermediates and deletion analysis located the origin required for the initiation of the plasmid vegetative replication, *oriV*, (Meyer and Helinski, 1977; Thomas et al., 1980) while *trfA*, which encodes the essential trans-acting Rep protein, is located upstream from *oriV* (Thomas et al., 1980).

RK2 *oriV* DNA contains 17 bp (of which 13 bp TGACANNTGAGGGGC are particularly conserved) direct repeats or "iterons" (Fig. 1), which, like iterons in other plasmids, play a very important role in both initiation of replication and regulation of plasmid copy-number by nucleo-protein complex formation with the plasmid-encoded replication initiation protein (TrfA) (Stalker et al., 1981; Pansegrau et al., 1994; Kolatka et al., 2010; Konieczny et al., 2014). The iteron sequence, orientation, spacing and bases between iterons are very important for TrfA binding and stability of TrfA nucleoprotein complexes: a complex of TrfA with one iteron was found to be highly unstable but co-operativity occurs between protein molecules in binding appropriate groups of iterons (Perri and Helinski, 1993). In addition to the 17 bp iterons, the RK2 minimal origin contains 9 bp sequences which are binding sites for DnaA, the bacterial chromosome replication initiation protein. The RK2 DnaA binding sequences conform closely to the *E. coli* DnaA box consensus sequence in chromosomal DNA (Fuller et al., 1984; Schaper and Messer, 1995) and binding of *E. coli* DnaA protein to all four sites has been confirmed experimentally (Konieczny et al., 1997). The inability of DnaA proteins from *Bacillus subtilis* and *Streptomyces lividans* to form a stable and functional complex with the DnaA boxes at *oriV*$_{RK2}$ has been proposed to be a limiting factor for plasmid host range (Caspi et al., 2000). RK2, like some other plasmids, also has a binding site for integration host factor (IHF) (Fekete et al., 2006; Konieczny et al., 2014).

Besides the group of five essential iterons located within the *oriV* sequence, there are two orphan singleton iterons (1 and 10) and an uneven triplet of iterons 2 to 4 (Pansegrau et al., 1994; Larsen and Figurski, 1994). Iterons 1 and 10 have identical sequences but face in opposite orientations, 262 bp upstream and 490 bp downstream from the 5-iteron cluster (Larsen and Figurski, 1994). Replication control of many plasmids is mediated by the balance between the positive and negative effects of the Rep protein binding to iterons in or around the replication origin, *oriV* (Chattoraj, 2000; Das and Chattoraj, 2004). Negative control is mediated by dimeric TrfA linking iterons in a process termed "handcuffing" (Blasina et al., 1996). Iterons 1–9 are not identical: positions 6 and 7 can be CT (iterons 1, 3, 5, and 7), GA (iterons 2, 4,

Fig. 1. Nucleotide sequence of oriV showing features relevant to this paper. The maximum *oriV* region present in pCT549 + iteron 10 starts at coordinate 11,742 on the RK2 DNA sequence from Genbank accession BN000925.1. The deletions marked P1, P1A, P1B and P1C remove flanking segments outside iteron 10. The essential iterons (5 to 9) are highlighted in green while the regulatory iterons (1 to 4 and 10) are highlighted in cyan. The start of the *klc* operon and KorA operator (OA highlighted in red) is included to show the proximity to *oriV*. DnaA binding sites between iteron 4 and 5 are also marked. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

6, 8), or TT (iteron 9) (Pansegrau et al., 1994). The "upstream" (defined with respect to the sequence with iterons in numerical order 1 to 9) 6 bp are non-random and, along with the 15 bp iteron, constitute the TrfA binding unit with TrfA having greatest affinity for iterons with the Py-Pu-Py motif immediately upstream (Perri and Helinski, 1993). Iterons 1 and 10 uniquely have the sequence TTTCAT upstream of the iteron: iteron 1 has been shown by footprint analysis to bind TrfA (Pinkney et al., 1988). Because iterons 1 and 10 have CAT at this location, they are likely to be high affinity TrfA binding units and because they are inverted, it was proposed that they may form a loop (Larsen and Figurski, 1994). Spacings between iterons and disturbances in the position of iterons relative to other origin region motifs, especially those that change helical phasing, can negatively influence plasmid replication (Shah et al., 1995; Doran et al., 1998; Das and Chattoraj, 2004; Konieczny et al., 2014). So, the present study was undertaken to understand the roles of these non-essential iterons in controlling RK2 plasmid copy-number using a mini-RK2 plasmid pCT549 (Thomas et al., 1980; Stalker et al., 1981; Thomas, 1981; Thomas et al., 1981; Durland and Helinski, 1990; Jagura-Burdzy and Thomas, 1992) as a model system.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The standard *E. coli* strains used were DH5α (Hanahan, 1983) and C2110 (*polA1 his rha*). Curing of plasmid F was tested in JM109 [pUC18] (Yanisch-Perron et al., 1985; Vieira and Messing, 1982). Wild type RK2 was used as template for *oriV* amplification. pCT549 (Thomas and Hussain, 1984; Fig. 2) was used as the starting point for mini RK2 plasmid constructions. Plasmid pDS3 (Thomas, 1981) was used as an internal reference for relative copy-number determination since it is smaller than any of the derivatives tested here, carries a different selectable marker (the chloramphenicol acetyl transferase gene, *cat*) and has copy number that gives a band intensity in the same range as the mini-RK2 plasmids. pGEM-T Easy (Promega) and pUC18 (Vieira and Messing, 1982) were used as cloning vectors. Bacteria were grown in L-broth (Kahn et al., 1979) at 37 °C with shaking at 200 rpm or on L-agar (1.5% *w/v*). Antibiotics tetracycline (Tc), chloramphenicol (Cm) and ampicillin (Ap) were added to a final concentration of 15, 25 and 100 μg ml^{-1} respectively.

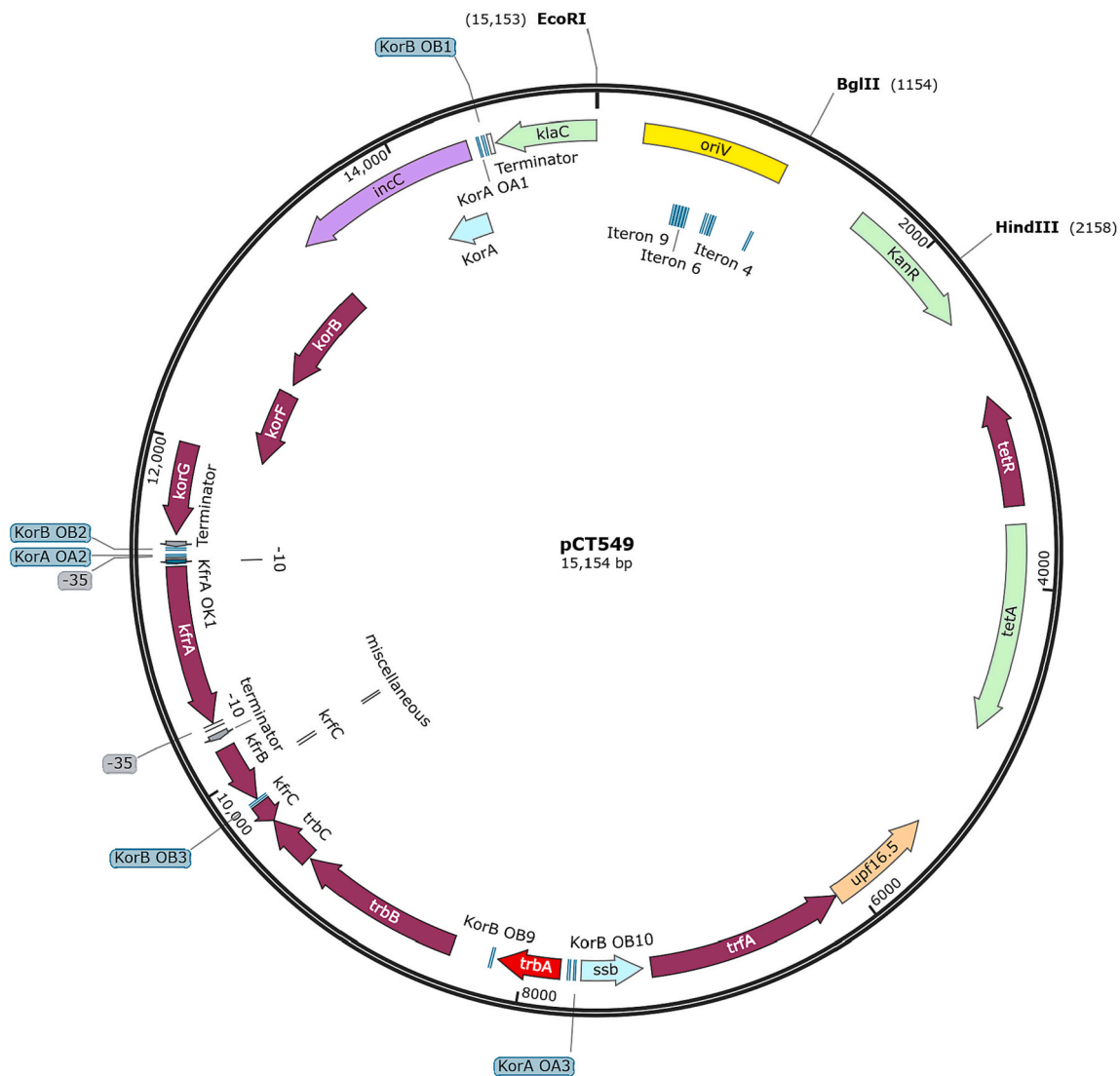**Fig. 2.** Map of the RK2 derivative pCT549 used as the starting point for the studies in this paper. KorA and KorB operators (OA and OB respectively) that mediate regulation of core backbone functions by the central control region are indicated. The central control region (*korA* to *korF*) and *kfr* operon provide basic partitioning and stable inheritance functions. The map was drawn in Snapgene (https://www.snapgene.com).

2.2. DNA extraction, manipulation and construction of mini RK2 plasmids

Plasmid DNA was extracted using a Bioline Isolate II Plasmid Mini Prep kit. Plasmid pCT549 (Fig. 2) was used as the starting point of these studies because it includes the complete central control and stability region. To allow insertion of the anti-F cassette, the linker consisting of forward and reverse strands shown in Table 1 which includes *Hind*III-*Pac*I-*Aat*II-*Bgl*II restriction sites was ligated between BglII and HindIII sites, inactivating the *aph* gene that confers kanamycin resistance and yielding pAPM1.

To amplify the different versions of *oriV* region of RK2 PCR was performed using the oligonucleotide primers (AltaBioscience, University of Birmingham, U.K and Sigma Aldrich) shown in Table 1 using Velocity Polymerase (Meridian Biosciences). Reactions were cycled in a SensoQuest Lab Cycler following standard procedures: initial denaturation, 98 °C for 2 min followed by 30 cycles of internal denaturation at 98 °C for 30 s, annealing at 5 °C below the Tm of the least stable primer determined using the Netprimer software (http://www.premierbiosoft.com/netprimer/) for 30 s before extension at 72 °C for 30 s per kb, with a final extension of 72 °C for 10 min. After amplification, the PCR products were purified using the Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE™ Healthcare).

Purified products were A-tailed using 6 μl of purified PCR products, 1 μl of Taq Buffer (Taq Polymerase, Invitrogen, United States), 1 μl of dATPs (Bioline), 1 μl of Taq Polymerase (Invitrogen, United States) and 1 μl of MgCl$_2$ (Invitrogen, United States). After A-tailing of purified products, they were ligated into pGEM-T Easy vector (Promega, United States). Ligated products were transformed into *E. coli* DH5α competent cells and selected on L-agar with X-Gal (50 μg ml^{-1}), IPTG (1 mM) and Ampicillin. Plasmid DNA was prepared from transformants and the inserts confirmed by DNA sequencing on an ABI 3730 DNA analyser (Functional Genomics, Proteomics and Metabolomics Facility, University of Birmingham, U.K.). To delete iterons 2–4 the segments of *oriV* on either side were amplified using primers F1/R4 and F10/R1, purified and then joined by Spliced Overlap Extension (Horton et al., 1990) and PCR before cloning in pGEM-T Easy.

After confirmation by DNA sequencing, the pGEM-T Easy derivatives as well as pAPM1 were digested with *Bgl*II and ligated overnight at 4 °C. The ligated products were transformed into *E. coli* C2110 which is PolA

deficient (to prevent the pGEM-T Easy plasmid from replicating) and selected on L-agar with tetracycline and ampicillin. Plasmid DNA was extracted from the transformants and digested with *Eco*RI to check the orientation of the insertion so that clones that would allow the high copy vector to be deleted by *Eco*RI digestion could be chosen. After *Eco*RI digestion, enzyme was heat inactivated at 65 °C for 20 min and then the digested products were ligated directly and left overnight at 4 °C. The products were then transformed into *E. coli* DH5α competent cells and selected on L-agar with tetracycline. Transformants were screened for loss of ampicillin resistance to confirm loss of the pGEM T Easy vector segment. Plasmid was prepared from three separate colonies for restriction digest confirmation with *Eco*RI and BglII (confirms the *oriV* of RK2). The hexamer mutants were constructed in a similar way except that the R3 primer was used so that the PCR product included a *Hind*III site which allowed insertion into pUC18 before substitution into pAPM1.

2.3. Relative plasmid copy-number determination

Multiple clones of RK2 derivatives were transformed into competent DH5α (pDS3) prepared from a culture grown from a single colony to minimize copy-number diversity. Transformants were selected on L-agar Tc Cm plates. At least three colonies for each clone were grown overnight in universal bottles with 5 ml L broth with tetracycline and chloramphenicol to minimize plasmid loss. Plasmid DNA was isolated as described above and eluted in 50 μl of elution buffer. Aliquots (5 μl) of each sample were digested with *Eco*RI to linearise the plasmid. Sample order was permutated and samples were separated by 1% (w/v) agarose gel plus ethidium bromide (0.1 μg ml^{-1}) and illuminated with a BioRad UV transilluminator imaging system. Band intensities were determined by creating a rectangle that could encompass the biggest area band on the gel and copying this so that the same rectangle was used for three determinations for each lane – the RK2 derivative, a background rectangle and the pDS3 band. The pixels were captured and quantified using the Gel Image software (BioRad, USA). Background was subtracted from the two plasmid DNA bands and the ratio of RK2 derivative to pDS3 determined for each sample. These ratio data were collected into spreadsheets which can be found in Supplementary data (Tables S1- S4). The mean and standard deviation for each derivative was determined after checking that there was no apparent and consistent difference

Table 1
Oligonucleotides and PCR primers used in this study.

Name	Base sequence (5′-3′)[a]	EndoR
Linker F	5′agcttACGttaattaaATGTACgacgtcCTAa 3′	*Apa*I *Aat*II
Linker R	5′gatctTAGgacgtcGTACATttaattaaCGTa 3′	ApaI AatII
oriV full length with i10	F1: ATCgaattcCGGCCGTACCCGATTC	EcoRI
	R1: GAGATagatctAGCGTGGACTCAAG	BglII
Delta P1	F2: ATCgaattcTACCCGATTCTGCGGTTACAG	EcoRI
Delta P1A	F3: ATCgaattcGAACGCCAAGGGGGAGCG	EcoRI
Delta P1B	F4: ATCgaattcCGGCCGTTGAATGGTGATC	EcoRI
Delta P1C	F5: ATCgaattcTTGAATGGTGATCGCCATCAC	EcoRI
oriV + i10 + TTTCAT	F6: ATCgaattc**TTTCAT**TGACACTTGAGGGGCGTTTAG	EcoRI
oriV + i10 - TTTCAT	F7: ATCgaattcTGACACTTGAGGGGCGTTTAG	EcoRI
oriV - i10	F8: CATgaattcGTTTAGAGCGAGCCAGGAAAG	EcoRI
oriV - i1	R2: TGAagatctACCGCAGGGAAATTCTCGTC	*Bgl* II
oriV i10 + GAACAT	F6.1: ATTCTAGATACgaattc**GAACAT**TGACACTTGAGGGGCGTTTAG	EcoRI
oriV i10 + TTTTTC	F6.2: ATTCTAGATACgaattc**TTTTTC**TGACACTTGAGGGGCGTTTAG	EcoRI
oriV i10 + TTTTAT	F6.3: ATTCTAGATACgaattc**TTTTAT**TGACACTTGAGGGGCGTTTAG	EcoRI
oriV i10 + TTTCTT	F6.4: ATTCTAGATACgaattc**TTTCTT**TGACACTTGAGGGGCGTTTAG	EcoRI
oriV i10 + TTTCAC	F6.5: ATTCTAGATACgaattc**TTTCAC**TGACACTTGAGGGGCGTTTAG	EcoRI
*oriV*i10mutR	R3: GACTGAATCCGGTGAGAATGGC	–
oriV inv. i10	F9: ATCgaattcGCCCCTCAAGTGTCAATGAAAgtttagagcgagccaggaaag	EcoRI
oriV inv. i1	R4: GATagatctGCCCCTCAAGTGTCAATGAAAaccgcagggaaattctcgtc	Bgl II
oriV with delta i2-i4	R5: TGGCCCTCAcaggctcgatttcggccg	-
	F10: AATCGAGCCTgtgagggccaagttttccg	-

^a F and R refer to forward (left to right) and reverse (right to left) primers relative to the sequence in Fig. 1. Upper and lower case are used to emphasise the different sections of the primer.

between the clones chosen. The mean ratio was compared to the mean ratio of the two basic standards: pCT549.1 with full length *oriV* (starting at coordinate 11,742) that includes iteron 10 and parental pCT549 that has the original *oriV* defined by the *Hae*II site at coordinate 12,097. *t*-Test was performed in Excel to determine the significance of differences in mean relative copy-number with 0.05 being used as indicative of a significant difference.

2.4. Determination of segregational stability

Transformant clones that had been used for plasmid extraction for copy-number determination were used to inoculate L-broth with tetracycline and chloramphenicol to select for both plasmids and grown overnight at 37 °C. The cultures were then diluted 1:1000 into L-broth with just chloramphenicol so that segregants that had lost the IncP-1 plasmid would grow normally. These cultures were grown for 9 h and then diluted 5000-fold into L-broth with just chloramphenicol and grown overnight. These saturated cultures were then serially diluted 10^6-fold and 100 μl spread on L-agar with chloramphenicol before replica plating onto L-agar Cm Tc and L-agar Cm to determine the number of colonies lacking the IncP plasmid.

2.5. Determination of curing efficiency against F plasmid

E. coli JM109 has a chromosomal deletion removing the *lac* operon and carries an F′ *prolac* plasmid encoding *lacZ* with a deletion removing the beta-galactosidase alpha fragment. To create a simple blue/white screening test for F'prolac displacement, pUC18 was transformed into *E. coli* JM109 to complement the *lacZ* defect (Lazdins et al., 2020). Competent JM109 (pUC18) were then transformed with plasmids to be tested and selected on L-agar with tetracycline, ampicillin (to ensure that bacteria retain pUC18), X-Gal and IPTG.

3. Results

3.1. Iterons 1 and 10 are equally important for copy-number control

The downstream end of the *oriV* region in pCT549 was defined by a *Hae*II site at RK2 coordinates 12,097–12,102 (Fig. 1) because mini-RK2 plasmids were originally constructed by HaeII partial digestion (Thomas

Table 2
Relative copy-number of pCT549 derivatives with different *oriVs*.

Versions of *oriV* in pCT549	Iterons in *oriV*	Relative copy-number[a]	T-test versus full length WT[b]
1. WT RK2 nt 11742–12996		1	
2. ΔP1 RK2 nt 11749–12996		1.09	0.926NS
3. ΔP1A RK2 nt 11812–12996		1.17	0.284NS
4. ΔP1B RK2 nt 11830–12996		0.82	0.165NS
5. ΔP1C RK2 nt 11835–12996		1.13	0.292NS
6. TTTCAT i10 nt 11858–12996		0.87	0.440NS
7. GAATTC i10 nt 11864–12996		1.53	0.027*
8. Δi10 ie pCT549 nt 11880–12996		1.45	0.012*
9. +i10 Δi1 nt 11742–12927		1.48	0.010**
10. inverted i10		0.77	0.0704NS
11. inverted i1		0.72	0.042*
12. inverted both i1 and i10		1.18	0.401NS
13. Δi1 + inv i10		1.66	0.0006*** but 0.25 v delta i1 + i10
14. Δi2–4		2.09	0.0002***
15. Δi2–4 Δi10		3.72	0.0003***
16. Δi2–4 inv i10		1.78	0.003**
17. Δi2–4 inv i1		1.49	0.017*
18. Δi2–4 Δi1 inv i10		3.6	3.8×10^{-6}****
19. Δi2–4 inv i1 Δi10		1.93	0.0009***

[a] This Table is based on the data in Supplementary Data Tables S1 to S4. SD is not shown since the statistics are based on the ratio with internal control not relative copy number.
[b] Significance is indicated: * $p \leq 0.05$; **, $p \leq 0.01$.

et al., 1980). To add the extra "downstream" sequences to introduce the "full" *oriV* region including iteron 10 we used PCR to amplify *oriV* from RK2 coordinate 11,742 just upstream of the KorA operator sequence associated with the *klcA* promoter (the start of the *klcA-klcB-korC* operon), the first downstream non-*oriV* region on the RK2 map. The other end was defined by the *Bgl*II site so that the PCR product could be inserted as an *Eco*RI to BglII fragment. Although iteron 10 is the obvious feature in this extra segment, it also contains a number of very G + C-rich tracts including repeated CGGCCG hexamers (Fig. 1). To determine whether this region also influences copy-number, we made five deletions (named P1, P1A, P1B, P1C and TTTCATiteron10 Fig. 1) and determined their relative copy-numbers (Table 2, data lines 2 to 5). While these deletions appeared to have minor effects on copy number none were statistically significant suggesting that the flanking region outside of iteron 10 and its associated hexamer does not contain any major copy-number control elements.

However, Larsen and Figurski (1994) noted that iteron 10 and iteron 1 share an identical adjacent 6 bp sequence (5' TTTCAT 3') so we made a pair of deletions, one that removes the TTTCAT of iteron 10 (putting an EcoRI site GAATTC in its place which changes every base) but leaves the iteron sequence intact and a second that removes the whole iteron as well. Remarkably, removal of just the TTTCAT sequence adjacent to iteron 10 causes as big a rise in copy-number as removing it along with the whole iteron and there is no further rise in copy-number when the DNA up to the HaeII site at nucleotide 12,097 is removed (Table 2, data lines 7 and 8). This demonstrates conclusively that iteron 10 and its adjacent sequence are parts of the key control element downstream of *oriV*. The effect on copy-number of removing iteron 1 is essentially identical to removing iteron 10 (Table 2, data line 9), reinforcing the conclusion that iteron 1 and 10 play similar roles as single iterons.

3.2. The effect of inverting iterons 10 and 1

The inverted orientation of iteron 10 relative to the other iterons and the presence of the extended sequence 5' TTTCAT 3' in iterons 1 and 10 prompted Larsen and Figurski (1994) to propose that iterons 1 and 10 work together by promoting the formation of a DNA loop mediated by the known DNA binding of TrfA (Kittell and Helinski, 1991; Blasina et al., 1996). To test this hypothesis, we therefore inverted iteron 10 and iteron 1 separately, predicting that in both cases copy-number should rise if their inverted orientation relative to each other is critical for their function. It is important to note that the sequence flipped included the adjacent 5' TTTCAT 3' sequence. To our surprise we found that inverting iteron 10 resulted in no statistically significant change in relative copy-number compared to wild type. However, we found that inverting iteron 1 caused a statistically significant drop (to 0.72) suggesting that the drop on flipping iteron 10 (to 0.87) may be real. Taken together these data suggest that if iteron 1 and 10 work together, they do so more efficiently when in the same orientation rather than when inverted. Indeed, when we flipped both iterons 1 and 10 so that they run once again in opposite directions, the relative copy-number rose to 1.18 (Table 2). Thus, these data do not support the hypothesis that iterons 1 and 10 are inverted relative to each other so they can form the strongest loop essential for blocking replication.

3.3. Influence of iteron 10 and 1 when iterons 2–4 are absent

We previously observed that deletion between iterons 2 and 4 to leave just one iteron at this location had a similar effect on average copy-number to deleting iteron 1 (Thomas and Hussain, 1984). We thought that looking at the effect of the lone iterons when iterons 2–4 are absent might be illuminating. Just deleting iterons 2–4 resulted in a doubling of relative copy-number which is greater than deleting either iteron 1 or iteron 10 alone (Table 2, line 14). Deleting iteron 10 in addition to iteron 2–4 resulted in almost a further doubling of copy-number (Table 2, line 15) which is consistent with previous observations with iteron 1

(Thomas and Hussain, 1984). Perhaps most significantly, inverting iteron 10 or 1 when iterons 2 to 4 were missing resulted in falls in copy-number (Table 2, lines 16 and 17) consistent with the falls seen when just iteron 10 or 1 were flipped with the rest of *oriV* complete. The relative changes in copy-number associated with these iteron inversions ($1.78/2.09 = 0.85$ and $1.49/2.09 = 0.71$) were almost exactly the same as observed without the iteron 2 to 4 deletion (Table 2, lines 10 and 11). Interestingly, deleting iteron 1 when iteron 10 was inverted had a much greater effect (Table 2, line 18) than deleting iteron 10 when iteron 1 was inverted (Table 2, line 19). The most obvious conclusion from this is that flipping iteron 1 has a major influence on the effect of other deletions and suggests that iterons 1 to 4 form a potent element or group of elements in replication control irrespective of the presence of iteron 10 and therefore that an interaction between inverted iterons 1 and 10 may not be important for their primary role.

3.4. Mutagenesis of the adjacent hexamer

In our construction with the adjacent essential TTTCAT hexamer sequence of iteron 10 "removed" it is replaced by an *Eco*RI site (GAATTC) which is quite a good match to the consensus proposed for this region by Perri and Helinski (1993) (NPuNPyPuPy). To check which part of this sequence is particularly important, five more derivatives were made with variations on this sequence (GAACAT, TTTTTC, TTTTAT, TTTCTT and TTTCAC; Tables 1 and 3). The data in Table 3 reveal that copy-number of the mutant plasmids tested vary between approximately 1 (the same as the WT with all ten iterons) and about 1.5 to 1.6 (the same as after complete removal of iteron 10). The data also showed that: a purine at position 2 is not needed but that all or part of the TTT motif (positions 1–3) is important for full activity; the T at position 6 is critical; positions 4 and 5 are less important.

3.5. Segregational stability of derivatives with reduced copy-number

Since some of the derivatives constructed appeared to have reduced copy-number, we considered the possibility that this might increase segregational instability, so this was determined as described in Materials and Methods. The results (Table 4) showed that most of the plasmids showed a low level of instability and that very few of the differences relative to the highest copy number plasmid tested, pCT549 (without iteron 10), which was >98% stable, were statistically significant. The low instability is probably related to the fact that, although these plasmids have the central control and partitioning region including the essential *incC* and *korB* (*parA* and *parB*) functions, they lack the complete set of other coregulated functions such as the *klc*, *kle*, *kla* and *kfr* operons that seem to act in an auxiliary way (Macartney et al., 1997; Haines, 2001). Possibly of most interest is the increased segregation rate observed for the two derivatives with iteron 1 flipped and deletion P1B which removes the first of at least three runs of GC

Table 3
Mutagenesis of the iteron-associated hexamer.

	Hexamer sequence	Relative Copy-number[a]	t-Test v WT[b]
WT	TTTCAT	1.0	
ΔTTTCAT	GAATTC	1.53	$P = 0.027$*
Mut1	GAACAT	1.28	$P = 0.016$*
Mut2	TTTTTC	1.53	$P = 0.0015$**
Mut3	TTTTAT	1.02	$P = 0.46$NS
Mut4	TTTCTT	0.89	$P = 0.23$NS
Mut5	TTTCAC	1.58	$P = 0.096$NS
Previous motif[c]	NRNYRY		

[a] This is based on data in Supplementary Data Table S5. SD is not shown since the statistics are based on the ratio with internal control rather thant relative copy number.

[b] Significance is indicated: * $p \leq 0.05$; **, $p \leq 0.01$.

[c] From Perri and Helinski, 1993, based on iterons 2 to 9.

Table 4
Loss rate of pCT549 derivatives over 22 generations based on data in Table S6.

pCT549 derivatives from Table 2	Relative copy-#	Mean % ± SD[a] Tc^{R}Cm^R/Total Cm^R	T-Test v pCT549-i10	Loss rate/ generation
pCT549.1 + i10	1.0	96.0 ± 3.7	P = 0.13NS	0.2%
pCT549 (no i10)	1.45	98.1 ± 2.0		0.1%
pCT549.9 inv. i1	0.72	89.1 ± 3.5	P = 0.00014***	0.5%
pCT549.8 inv. i10	0.87	99.0 ± 1.3	P = 0.21NS	0.05%
pCT549.9 inv1 + i10	1.18	89.1 ± 11	P = 0.097NS	0.5%
pCT549.3 ΔP1	1.09	98.4 ± 1.4	P = 0.40NS	0.07%
pCT549.4 ΔP1A	1.17	97.5 ± 3.4	P = 0.40NS	0.1%
pCT549.5 ΔP1B	0.82	84.3 ± 4.5	P = 8 × 10^{-6}****	0.8%
pCT549.6 ΔP1C	1.13	96.4 ± 0.6	P = 0.039*	0.17%

[a] This is based on data in Supplementary Data Table S6.

pairs. To check that altered stability was not due to a perturbation of monomer/dimer ratio or a change in topology we analysed uncut plasmid DNA of each of these plasmids by agarose gel electrophoresis but saw no detectable differences in the profile of the DNA (See supplementary Fig. S3). Further analysis of segregation instability may therefore be warranted but is beyond the scope of this paper.

3.6. Potentiation of curing by different versions of oriV

Although the potentiation of displacement of F'prolac by our IncP vectors correlated with the presence of the regions with iterons 1 and 10, the rise in copy-numbers when these are deleted is not large and so it is possible that there is another explanation. Therefore, representative constructions that showed a range of relative copy-number were used as the vectors for the anti-F cassette and displacement of F'prolac was assessed as described in Methods using the blue-white screening provided by the presence of pUC18 (Table 5). The results showed that when all 10 iterons are present in *oriV* with the AntiF cassette in the plasmid, essentially no curing is observed (<2% loss of F after transformation with the cassette-bearing plasmid), but deletion of either iteron 1 or iteron 10 in *oriV* of RK2 with AntiF potentiated curing dramatically (>99%). Similarly, when copy control iterons 2–4 were deleted full curing was observed (>99%) (Table 5). However, the minor variation in copy-number as the region adjacent to iteron 10 was deleted (deletions P1 to P1C) made no difference.

Table 5
Efficiency of F-plasmid displacement by pCT549 derivatives.

Plasmid from Table 2	*oriV* iterons present	Anti-F	Colonies (Blue/White)	% Curing rate: – F'pro loss
pCT549.1	iterons 1–10	–	Blue	<1
pCT549.1.aF	iterons 1–10	+	Blue	<2
pCT549.3.aF	iterons 1–10 ΔP1	+	Blue	<2
pCT549.7.aF	iterons 1–10 ΔP1A	+	Blue	<2
pCT549.8.aF	iterons 1–10 ΔP1B	+	Blue	<2
pCT549.9.aF	iterons 1–10 ΔP1C	+	Blue	<2
pCT549	iterons 1–9	–	Blue	<1
pCT549.aF	iterons 1–9	+	White	>99
pCT549.2.aF	iterons 2–10	+	White	>99
pCT549.13.aF	iterons 1, 5–10	+	White	>99
pCT549.14.aF	iterons 1, 5–9	+	White	>99

3.7. Iterons in oriV regions of all known IncP-1 subgroups

Given the importance of iteron 10 in replication in RK2, we surveyed other major IncP-1 subgroups for iteron 10 homologues. Table 6 shows that all IncP-1 subgroups tested do appear to have an iteron 10, but that conservation of the upstream hexamer is not consistent. This may suggest that the conservation of the hexamer with iteron 1 and 10 in RK2 is a coincidence, especially since Table 3 shows that some changes in hexamer sequence have no effect on iteron function. It is also possible that the copy-number of different IncP-1 plasmids has evolved to suit the context in which it has evolved. In addition, we discovered that a number of the other IncP-1 subgroups have an eleventh iteron, generally located at the downstream end of the *klcA* orf and so this may influence copy-number and could possibly affect the need for the hexamer adjacent to iteron 10.

a. The sub-groupings chosen are based on Shintani et al., 2020 although we recognise that additional complexity has been reported more recently (Hayakawa et al., 2022). The Eta subgroup is not included because although it has a *trfA* gene, the *oriV* iterons are not organised in the same way as the other sub-groups.

4. Discussion

The first critical point that we have established in this paper is that iterons 1 and 10 do not need to be inverted relative to each other to have a significant effect on plasmid copy-number. Indeed, our evidence suggests that when iterons 1 and 10 are in direct repeat they may have a slightly stronger negative effect on copy-number than when inverted relative to each other and this is observed both when i1 is flipped and potentially when i10 is flipped. This also shows that their effect does not depend on their orientation relative to the other groups of iterons, i2-i4 or i5-i10 as might be expected if TrfA dimers mediate looping between iteron 1 or 10 and the essential iterons. A caveat is that the distances between iteron 1 or 10 and the essential iterons (454 bp and 489 bp respectively between them and their nearer end of the iterons 5 to 9 group) are quite long relative to those studied in the P1 system by Das and Chattoraj (2004) where single iterons were added 16, 21, 26 or 31 bp from a group of five tandemly repeated iterons and a clear effect of orientation was seen, so in *oriV* of RK2 there is potentially much more flexibility. In the *gal* regulatory system where a loop is important for repression and both parallel and antiparallel are theoretically possible, there seems to be clear preference for the anti-parallel version (Virnik et al., 2003) but as shown in Fig. 3 this is feasible in both direct and inverted orientation. It would therefore be interesting to observe the structures of supercoiled mini-*oriV* plasmids with TrfA protein and HU which is known to facilitate loop formation and action of TrfA at *oriV* (Konieczny et al., 1997).

The second critical point is that we have confirmed that the hexamer

Table 6
Summary of "iteron 10s" in subgroups of IncP-1 plasmids.

| IncP-1 sub-group[a]	Plasmid example	i10?	Hexamers by i1 and i10	Extra iterons (i11)?
Alpha	RK2	yes	TTTCAT/TTTCAT	No
Beta-1	R751	yes	ACTCTT/GCTTCT	Yes, end of *klcA*
Beta-1	pB3	yes	ACTTCT/GCTTCT	Yes, end of *klcA*
Beta-1	R906	yes	ACCCTT/TTTCAT	Yes, end of *klcA*
Beta-2	pA1	yes	ACTCTT/TTTCTT	Yes, end of *klcA*
Beta-2	pB4	yes	CCCCTT/ CTGAAG	Yes, end of *klcA*
Gamma	pQKH54	yes	TTTCAT/ AGTCGT	Yes, but 10 + 11 together
Delta	pAKD4	yes	GCGTGC/ TTTCTT	Degenerate
Epsilon	pKJK5	yes	TTTCAT/TCATCT	Yes, end of *klcA*
Zeta	pMCBF6	Yes	No iteron 1/ CTTTAT	Yes, end of *klcA*
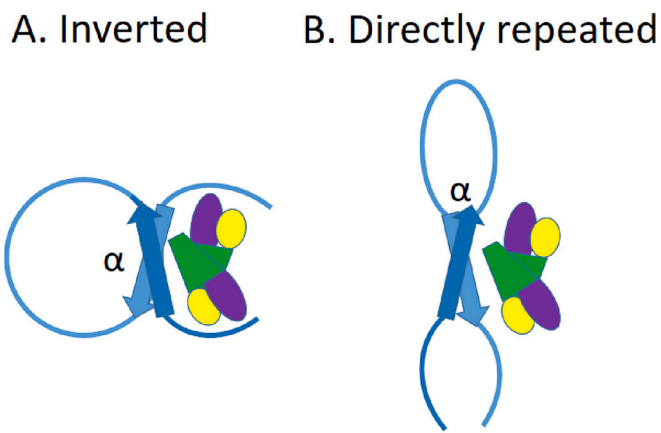
Fig. 3. Comparison of how inverted (A) and directly repeated (B) iterons (blue arrows) in part of a negatively supercoiled DNA molecule might align anti-parallel to allow binding of dimeric TrfA. Domains of TrfA are shown in purple (aa 98–193), green (aa 194–310) and yellow (aa 311–382) based on Wegrzyn et al. (2021) and the hypothesis that the green domain is responsible for dimerization since mutations G254D and S267L inhibit dimerization (Toukdarian and Helinski, 1998). Experimentally the two structures might be distinguished by the angle (α) between the DNA strands in the small circle formed (Virnik et al., 2003). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Logo motifs for the hexamer upstream of the RK2 TrfA iterons based on all or different subsets of iterons as indicated determined using Weblogo. Panel A uses iterons 2 to 9 which were used for the consensus established by Perri and Helinski (1993). Panel (B) shows the effect of including all 10 iterons while panels C and D show how distinct the logo is for iterons essential for control of copy-number (C) and *oriV* activation (D).

upstream of iteron 1 and 10 is very important in the negative action of these iterons and that the regulatory effect on plasmid copy-number can be wiped out by changing a single base pair, although some changes to the hexamer have no effect. Although it is well established that the hexanucleotide upstream of the TrfA iteron is needed for efficient binding (Perri and Helinski, 1993), the established consensus for this hexamer (NRNYRY) does not match the limited mutational analysis we present here (Table 3). Thus, the R at position 2 is clearly not essential for the action of iterons 1 and 10 since replacement of the nucleotides 1 to 3 (TTT) by GAA, which matches the consensus (NRN) better, resulted in partial loss of replication control. Similarly, while positions 4 to 6 (CAT) match the consensus (YRY) very well, changing this to CAC, which still matches the consensus, resulted in loss of control. Additionally, changing CAT to CTT, which does not match the consensus, appeared to strengthen control, suggesting that the selection pressure that favours particular bases at each position is not simply favouring strongest binding or that the consensus is wrong.

Since iterons 1 and 10 were not included in the analysis that generated this consensus, we created a logo (Schneider and Stephens, 1990; Crooks et al., 2004) based on the hexamer sequences upstream of the iterons (2 to 9) used by Perri and Helinski (1993) and then all ten iterons to help visualise the sequence requirements (Fig. 4A and B). Rather than helping to identify selected positions, the logo for the full set (iterons 1 to 10, Fig. 4B) was very disappointing because features visible in the logo for iterons 2 to 9 became weaker: it did not identify any strong selection at positions 1 to 3 but did suggest weak selection for YRY at positions 4 to 6. However, since the logo for iterons 2 to 9 (Fig. 4A) does show stronger selection at positions 1 to 3 this suggested that Iterons 1 and 10 may be under different selective pressure, which may not be surprising since their role appears devoted to control rather than activation of replication.

To test this hypothesis further we created separate logos for: iterons 1 to 4 and 10 (Fig. 4C), which are not needed for origin activation but are associated with copy-number control via TrfA dimers; and for iterons 5 to 9, which are essential for activation of the origin by TrfA monomers (Fig. 4D)(Toukdarian et al., 1996). Remarkably, these two logos show strong selection at multiple positions (1, 2, 4, 5, 6) and, most significantly, look distinctly different from each other with a particularly dramatic difference at position 4 where the 5 to 9 logo is almost all T
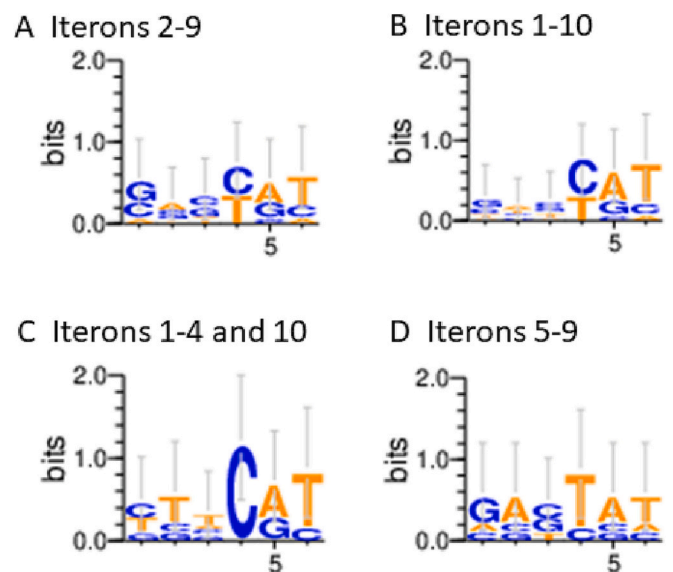
while the 1 to 4 and 10 logo is entirely C. This suggests that these two sets of iterons might show different binding affinities for TrfA monomers and dimers which could be tested experimentally. Dimeric TrfA can be purified from a strain expressing mutant *trfA* (S257F) (Toukdarian and Helinski, 1998) while monomeric TrfA can be made from a copy-up mutant that suppresses dimer formation TrfA(G254D/S267L) (Blasina et al., 1996; Durland et al., 1990). The study of Perri et al. (1991) observed only weak TrfA binding with plasmids pSP7 and pSP9 carrying single iterons 2 and 8 respectively (in our nomenclature), which have the hexamers 5'-CCTCAC-3' and 5'-AGATGA-3', but both lack the T at position 6 which seems to be critical for full activity in copy-number control (Table 3). It may also be that the spacing of the activating iterons favours the cooperative binding of monomeric TrfA while regulatory iterons may be spaced to reduce cooperativity and allow hand-cuffing to be reversed relatively easily.

We also created logos for the two sub-sets of iterons from a selection of plasmids representing different subgroups of IncP-1 plasmids (Fig. 5). This showed evidence of selection within these sequences and differences between the subsets for most plasmids. However, the actual selected positions and nucleotides dominating at these positions were not consistent across all the plasmids suggesting that there is diversification of this function across the family. This could be one basis for weakened incompatibility between plasmids of different IncP-1 subgroups.

Although the presence of multiple regulatory iterons must give a degree of robustness to the copy-number control, the fact that a single SNP can result in a copy-number change that can alter phenotype significantly, as illustrated by our observed change to plasmid curing and to kanamycin resistance MIC (Lazdins et al., 2020), demonstrates both the potential of this system for adaptation to modulate competition with related plasmids and also to change the intensity of phenotypes conferred by dosage-dependent genes, such as those encoding beta-lactamases or aminoglycoside phosphotransferases. It also emphasises the ability of the regulatory and activating properties of *trfA/oriV* to evolve quasi-independently. Comparison of the copy-numbers of different members of the IncP-1 subfamily and correlating these with the sequence of their replication origins and their phenotypic load might provide a useful foundation for analysing plasmid sequences from novel
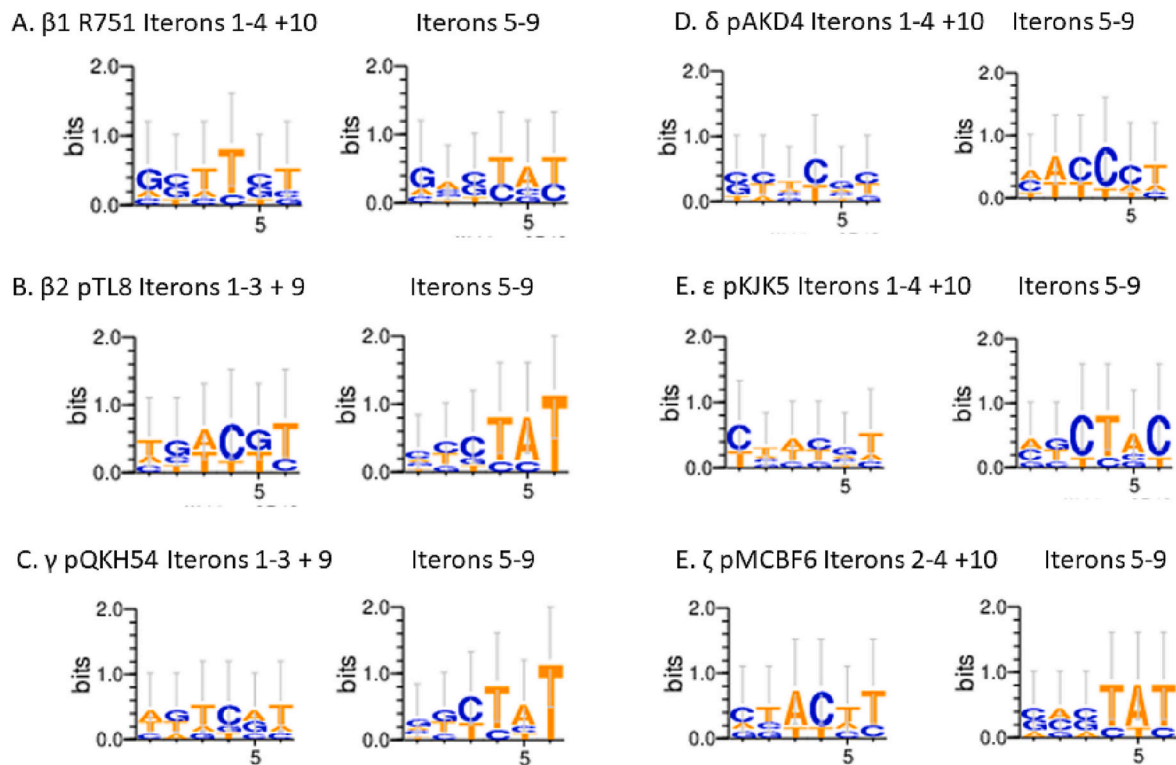
Fig. 5. Logos for regulatory and activating iterons from selected individual plasmids in different IncP-1 subgroups β1, β2, γ, δ, ε, ζ. This analysis is not meant to be comprehensive but demonstrates that not all IncP plasmids show the same hexamer motifs nor the marked difference between regulatory and activating iterons observed in IncPα plasmids.

isolates from clinical and environmental sources.

Data availability

All data underpinning the statistics are provided in Supplementary data. Gel images and Quantity One data from those images will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plasmid.2023.102681.

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