Evolution of the *korA*–*oriV* segment of promiscuous IncP plasmids

Christopher M. Thomas, Christopher A. Smith,[†] John P. Ibbotson,[‡] Lynda Johnston[§] and Naijin Wang^{||}

Author for correspondence: Christopher M. Thomas. Tel: +44 121 414 5903. Fax: +44 121 414 5925.

¹ School of Biological Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Plasmids belonging to *Escherichia coli* incompatibility group P are of particular interest because they can transfer between, and be stably maintained in, almost all Gram-negative bacterial species. The segment of the IncP α plasmid genome between the key regulatory gene *korA* and the vegetative replication origin, *oriV*, encodes a series of operons co-regulated with replication and transfer functions by the KorA protein. To determine which of these genes are likely to have an important role in IncP plasmid survival the equivalent region of the distantly related IncP β plasmid R751 was sequenced. Sequence comparisons show that the *kla* operon (formerly the *kilA* locus, which is also responsible for a cryptic tellurite-resistance determinant) is completely absent from R751. Similarly in the *kle* region, which encodes genes associated with the Kile⁺ phenotype of unknown function, *kleC* and *kleD*, which we proposed arose by a duplication of *kleA* and *kleB*, are also completely absent. The genes that are conserved are *kicA* (formerly *kilC*, responsible for the KilC⁺, and recently proposed to be involved in overcoming restriction barriers during transfer), *kicB* (an ORF interrupted by Tn1 insertion in RK2), *korC* (a transcriptional repressor which controls the *kicK* and *kle* operons), and *kleA*, *kleB*, *kleE* and *kleF*. A striking feature of the organization in R751 is the lack of the strong transcriptional termination signals which are present in IncP α plasmids. The degree of divergence between the plasmids facilitates the identification of motifs of probable functional importance in the primary protein sequences.

Keywords: broad-host-range plasmid, plasmid R751, genome evolution, plasmid maintenance, antirestriction

INTRODUCTION

Considerable attention has been focused on plasmids belonging to *Escherichia coli* incompatibility group P because of their ability to transfer between, and maintain themselves stably in, diverse species of Gram-negative

bacteria (for a review see Thomas & Smith, 1987). Most plasmids of this group fall into one of two subfamilies, IncP α or IncP β . The best-studied IncP α plasmids form an indistinguishable group of plasmids, RK2, RP1, RP4, R68 and R18 (Pansegrau *et al.*, 1994) (Fig. 1). In this paper, the names RK2 and RP4 will be used because the IncP α segments studied have been derived from both isolates. Detailed molecular genetic analysis of RK2 and RP4 has defined the blocks of genes required for vegetative replication and conjugative transfer as well as the genes involved in segregational stability (Thomas & Helinski, 1989; Guiney & Lanka, 1989; Pansegrau *et al.*, 1994). For vegetative replication the plasmid requires *oriV*, the replication origin (Figurski & Helinski, 1979; Thomas *et al.*, 1980; Stalker *et al.*, 1981), and *trfA*, which encodes the initiator protein, TrfA (Figurski & Helinski, 1979; Thomas *et al.*, 1980; Shingler & Thomas, 1984a; Smith & Thomas, 1984). For conjugative transfer the

[†] **Present address:** Department of Biological Sciences, Keele University, Keele, Staffordshire ST5 5BG, UK.

[‡] **Present address:** Department of Infection, University of Birmingham Medical School, Edgbaston, Birmingham B15 2TT, UK.

[§] **Present address:** Promega, Delta House, Enterprise Road, Chilworth Research Centre, Southampton SO1 7NS, UK.

^{||} **Present address:** Department of Biochemistry and Physiology, University of Reading, Whiteknights, Reading RG6 2AJ, UK.

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plasmid requires *oriT*, the transfer origin (Guiney & Helinski, 1979; Guiney & Jakobson, 1983; Pansegrau *et al.*, 1990), as well as proteins encoded in the *traJ-traA* operon (Lessl *et al.*, 1993), the *traK-traM* operon (Guiney *et al.*, 1989) and the *trb* operon (Lessl *et al.*, 1992).

A unique feature of the IncP plasmid organization is the coordinated expression of these genes for replication and transfer that is mediated by regulatory proteins encoded in the central control operon (*korABF*) (Motallebi-Veshareh *et al.*, 1992; Thomson *et al.*, 1993) (Fig. 1). The product of *korB* is known to repress transcription of both the *trfA* and *trb* operons (Shingler & Thomas, 1984b; Motallebi-Veshareh *et al.*, 1992; Thomson *et al.*, 1993; Williams *et al.*, 1993), while multiple binding sites for KorB are found in both the *tra* and *trb* regions (Balzer *et al.*, 1992), although whether KorB modulates gene expression when it binds at these points is unknown.

The *korA* gene product also acts at many points, but all of these are known promoter regions. Some of these regions coincide with promoters repressed by KorB (*korAp*, *kfrAp*, *kilAp* and *trfAp*) (Shingler & Thomas, 1984b; Smith *et al.*, 1984; Schreiner *et al.*, 1985; Young *et al.*, 1985, 1987; Theophilus *et al.*, 1985; Thomas *et al.*, 1990). At the face-to-face divergent promoters *trfAp* and *trbAp*, KorA acts both negatively and positively, repressing *trfAp* and simultaneously derepressing *trbAp* (Jagura-Burdzy & Thomas, 1994). The other promoters under KorA control are repressed by a third repressor, KorC, but not KorB (*kcrAp*, *kcrBp* and *kilCp*) (Thomas *et al.*, 1988; Kornacki *et al.*, 1990, 1993). It has been suggested that the products of the operons which are co-regulated with replication and transfer functions, but do not encode genes essential for either of these processes, are likely to play an auxiliary role in plasmid maintenance and propagation (Figurski *et al.*, 1982; Larsen *et al.*, 1994). Although no clear role has yet been established for stability systems, the IncP plasmids appear to have systems which have not yet been mapped to specific loci and may turn out to be encoded by these 'cryptic' operons (Harayama *et al.*, 1980; Jovanovic *et al.*, 1994).

One way of testing this hypothesis is to exploit the fact that the IncP family has two branches which have undergone considerable divergence from their common ancestor but have retained many fundamental replication, transfer and control functions intact (Lanka *et al.*, 1985; Meyer & Shapiro, 1980; Figurski *et al.*, 1982; Villaroel *et al.*, 1983; Chikami *et al.*, 1985; Jakobson & Guiney, 1983). The best-studied IncP β plasmid is R751 (Jobanputra & Datta, 1974) (Fig. 1). Sequence comparisons have already shown that considerable drift has occurred where the sequences are under less constraint (Smith & Thomas, 1985; Furste *et al.*, 1989; Miele *et al.*, 1991; Waters *et al.*, 1992; Ziegelin *et al.*, 1991). Therefore, conservation of function between IncP α and IncP β plasmids indicates that the gene provides a selective advantage for the plasmid. In this paper we present a comparison of the plasmid sector between *korA* and *oriV*. The results allow identification of the 'core' functions in this region which are present in both IncP subfamilies.

METHODS

DNA manipulations and sequencing. Plasmid DNA was isolated from *E. coli* C600 by the method of Birnboim & Doly (1979) for small-scale preparation and by a modified version of this method for large-scale preparation (Smith & Thomas, 1983). Manipulation and analysis of DNA was essentially as described by Maniatis *et al.* (1982). DNA sequencing was carried out by the method of Maxam & Gilbert (1980), with minor modifications (Smith & Thomas, 1987), and by chain-termination (Sanger *et al.*, 1977), using either a Sequenase 2 kit from USB or ABI kit with dye-terminators, followed by analysis on an ABI 373A automated DNA sequencer. Computer manipulations of the DNA sequence were carried out using the University of Wisconsin GCG package (Devereux *et al.*, 1984).

RESULTS AND DISCUSSION

The *korA-oriV* segment of R751 is shorter than that of RK2

We previously cloned the 9.8 kb *SaI* fragment of R751 running from coordinates 0 to 10 on the standard map (Fig. 1). We subcloned *oriV* from this and determined its DNA sequence (Smith & Thomas, 1985). Subsequently we have sequenced almost all of this *SaI* fragment. The segment which is the subject of this paper (GenBank accession number U08908) was located by identifying the position of the regulatory gene *korA* by searching for homology to the RK2 *korA* gene. The assumption that there would be a *korA* gene was based on the observation that R751 is phenotypically KorA⁺ (Figurski *et al.*, 1982). *korA* was located easily using the program GAP (Devereux *et al.*, 1984). By using *oriV* and *korA* as reference points we were able to determine that the distance between them is 4.3 kb. This contrasts with a distance of 12.7 kb for RK2 or 7.7 kb when Tn1 is excluded. Clearly there are additional sequences in this segment of RK2 which are not present in R751.

Alignment of R751 and RK2 sequences between *korA* and *oriV*

To compare R751 with RK2, the *oriV-korA* region of RK2 excluding Tn1 was sequenced. Our sequencing of parts of this region has been reported previously (Thomas *et al.*, 1988; Walter *et al.*, 1991), while the intervening regions have been deposited in the databases (*oriV-Tn1*, L13392; *klaA-klaA*, L13287), except for the Tn1-*korC* region, which had been published by others (M32794; Kornacki *et al.*, 1990). The *klaA-kleF*, *klaA-klaC* and *klaA* regions have also been sequenced (Kornacki *et al.*, 1993; Goncharoff *et al.*, 1991; Larsen & Figurski, 1994). Inspection of the RK2 DNA sequence on either side of the Tn1 insertion reveals a 5 bp duplication (TTTAA), which strongly indicates that there is a simple insertion with no associated deletions or rearrangements. This is confirmed by the comparison with R751, whereby the R751 sequences which line up with the RK2 sequence on either side of the Tn1 insertion are contiguous and show no evidence of loss of DNA from RK2 (data not shown). Comparison of the *oriV-korA* regions by a dotplot allowed us to locate regions of high conservation between

Fig. 1. Maps of RK2 and R751 showing the global regulatory circuits already established for RK2. The basis for the physical and genetic map of RK2 is given by Pansegrau *et al.* (1994). The alignment of RK2 and R751 is based on previous studies (Smith & Thomas, 1985, 1987; Smith *et al.*, 1993; Pansegrau & Lanka, 1987; Miele *et al.*, 1991; Waters *et al.*, 1992; Furste *et al.*, 1989; Ziegelin *et al.*, 1991); this work and unpublished data from this laboratory (C.M.T., Peter Thorsted, Chris Yates, Alex Foster and L.J.). Genes of known function are indicated as follows: replication, ▨; transfer, ▤; control, ■.

R751 and RK2 (Fig. 2). It is clear from these results that *klcA*, *klcB*, *korC*, *kleA* (*kcrA1*), *kleB* (*kcrA2*), *kleE*, *kleF* and *korA* are highly conserved genes. *klcA* and *klcB* are in the operon responsible for the KilC⁺ phenotype (Figurski *et al.*, 1982; Larsen & Figurski, 1994), which is expressed in the absence of *korC* (KilC override). *kle* genes belong to the region associated with the KilE⁺ phenotype (Young *et al.*, 1987). Analysis of the DNA sequence with the program CODONPREFERENCE showed that all the new ORFs identified in this segment of R751 conformed to the pattern of codon usage expected from known functional ORFs in RK2/RP4.

Major deletions/insertions

A number of gaps or insertions are revealed by the alignment of RK2 and R751 sequences in the *oriV*–*korA* region. First, there is an extra 600 bp upstream of *klcA* in R751 which has no significant sequence identity to the equivalent region of RK2 (Fig. 2a). Secondly, sequence similarity between R751 and RK2 disappears immediately after the end of *klcA* and does not reappear until about 400 bp later, relative to the RK2 sequence. On R751 the gap is only about 100 bp. In both RK2 and R751 there appears to be a long ORF which occupies the whole *klcA*–*korC* gap. However, this would have been disrupted in RK2 by the insertion of Tn1, which might have removed the constraint to maintain the integrity of this gene, allowing the drift which is observed in RK2 (Fig. 2a). Thirdly, there is a break in the diagonal where *kleC* and *kleD* are in RK2 (Fig. 2b), consistent with the proposal that the *kleA/B* and *kleC/D* operons in RK2 arose by duplication (Thomas *et al.*, 1988). There is a

second diagonal displaced to the right that shows *kleA* of R751 aligning with *kleC* of RK2, which was expected from the relatedness of RK2 *kleA* and *kleC* noted previously by Thomas *et al.* (1988). Comparison of *kleA* from RK2 and R751 with *kleC* from RK2 as well as the predicted polypeptide products (RK2 KlcA:R751 KlcA, 68%; RK2 KlcA:RK2 KlcC, 38%; R751 KlcA:RK2 KlcC, 36%) showed that the two *kleA* genes are more closely related than either is with *kleC*. Comparison of codon usage of conserved amino acids showed a much higher proportion of completely conserved codons between the two *kleA* genes than with the *kleA*–*kleC* pairs. One would expect that if the higher degree of conservation were due to functional constraints then drift would have allowed greater divergence in the third position of the codons for conserved amino acids. This strongly suggests that the separation of *kleA* and *kleC* preceded the separation of the α and β *kleA* genes. We therefore propose that this duplication was possessed by the common ancestor of the IncP plasmids, and that it has subsequently been lost by the IncP β subfamily. A fourth nonconserved region is described in the next section.

The *klaA**klaB**klaC* (*kilA**telA**telB*) region is completely absent from R751

In RK2, the *korA* gene is preceded by an operon which is regulated by KorA and KorB; the operon is associated with cryptic tellurite resistance and IncW plasmid fertility inhibition (Goncharoff *et al.*, 1991; Walter *et al.*, 1991; Yusoff & Stanisich, 1984). This is completely absent from R751 (Fig. 2b), which is consistent with the lack of cryptic tellurite resistance (Bradley, 1985). Downstream of *kleF*,

Fig. 2. Comparisons of the RK2 (horizontal axis) and R751 (vertical axis) *oriV-korA* segments. Dotplots were created using COMPARE with a window size of 20 and a stringency of 13. ORFs, solid blocks with open arrow heads; promoters, solid circles with arrow; terminators, t. The comparison was split into two based on the position of Tn1 insertion in *klcB* in RK2. (a) *oriV-klcB* (N-terminal region) segment. Repeats in *oriV* region are shown as bars. Two of the degenerate repeats downstream of *oriV* (numbers 10, 11 and 12) are inverted (repeat 10 and 12) relative to the orientation of the repeats in *oriV*. (b) *klcB* (C-terminal region) to *korA* segment.

RK2 and R751 diverge so that there is no trace of the *klaA* promoter/operator region. Sequence identity only returns about 300 bp further on with respect to the R751 sequence, at the *korA* promoter/operator region (Fig. 3a). These genes therefore have either been deleted from R751 or gained by RK2. The degree of similarity between the *korA* and *kilA* promoters of RK2 versus the degree of divergence between the *korA* promoters of RK2 and R751 (Fig. 4b) suggests that the two promoters must have arisen by a duplication event more recently than the divergence of the IncP α and IncP β subfamilies. However,

there are also considerable similarities between *korA* and *trfA* promoters within the IncP α subfamily despite the clear existence of both promoters in the common IncP ancestor, suggesting that selective pressure or gene conversion may maintain promoter similarities within a plasmid genome. This is borne out for the KorC-regulated promoters as described below. Therefore, we cannot make any deductions about whether the common ancestor contained the *kla* operon. However, we can conclude that the *klaA**klaB**klaC* operon is not essential for efficient survival of IncP plasmids.

Fig. 3. Alignment of sequences from RK2 and R751. (a) *korA* DNA sequences; (b) *KorA*-regulated promoters in which O_A overlaps the -10 region; and (c) *KorA* amino acid sequences. Solid lines indicate identity; colons indicate similarity. O_A and O_B sequences are the inverted repeats of the operators which mediate repression by *KorA* and *KorB*, respectively.

Fig. 4. Alignment of sequences of RK2 and R751 *KorC*-regulated promoters. Solid lines indicate identity.

The gap between *kleF* and *korA* in R751 contains an additional ORF of 89 codons. We have tentatively called this *kleG*, but we do not know whether it is expressed as a polypeptide. Database searches revealed no unambiguous similarities, but there was alignment with a variety of bacterial integrases, raising the intriguing possibility that in the IncP α ancestor this region could have promoted acquisition of a foreign DNA segment but may then have been lost in rearrangements to bring the new genes under the control of a promoter derived by duplication of the *korA* promoter.

Conservation of *oriV*-related sequences

We have previously reported that *oriV* sequences diverge significantly (about 35% mismatches) between RK2 and R751 (Smith & Thomas, 1985). Fig. 2(a) shows a grid-like pattern due to alignment of the iterons to which TrfA binds (Pinkney *et al.*, 1987; Kittell & Helinski, 1991), which are repeated nine times in the RK2 *oriV* region and eight times in the R751 *oriV* region (the ninth repeat in R751 is located on the other side of Tn4321). These repeats are followed by conserved A+T- and G+C-rich regions and then by a region in which we previously identified additional TrfA iterons in direct and in inverted orientation (Smith & Thomas, 1985, 1989). Each of the sequence features shows conservation with RK2. A very closely related sequence also appears at the end of *kleA* (TGACTGATGAGGGC in R751 compared to TGACA^{G/C}A/TGAGGGC in RK2 and R751 *oriV*), giving rise to the short repeated diagonals aligning between the end of *kilC* of R751 and *oriV* of RK2. The significance of these extra sites is not understood. It is possible that they modulate initiation negatively as proposed for the sites upstream of the minimal origin (Thomas *et al.*, 1984). Alternatively, they may assist in the activation of the replication origin by forming a TrfA-mediated loop as was proposed for RSF1010 (Haring & Scherzinger, 1989).

It was previously found that the *HaeII oriV* was not able to promote the multiplication of an M13-derivative which was defective in the lagging-strand origin, implying that a primosome assembly site, if it exists, must lie further downstream (D. G. Guiney, personal communication). Unfortunately, the sequence alignments did not reveal an obvious candidate for such a lagging-strand origin or primosome assembly site outside the *HaeII oriV* fragment.

Transcriptional terminators are not conserved

In RK2 there are very strong putative rho-independent transcriptional terminators located upstream of *korA* (Thomas & Smith, 1986) and *kleA* (*kcrA*) (Thomas *et al.*, 1988); these define the ends of *klaABC* and *klaABkorC* operons, respectively, and appear to protect the downstream operons from read-through transcription. Neither of these putative terminators is conserved in R751 and alternatives do not appear to exist. For *korAp*, the next upstream promoter is only 1.3 kb away. This gap includes the highly conserved hairpin between *kleB* (*kcrA2*) and *kleE*, but this is unlikely to be a transcriptional terminator.

korC is separated from the *klaA* promoter by about 2 kb. In RK2, both regions which now contain terminators have additional DNA which is not present in R751. In the case of the *klaAkorC* operon the *bla* gene of Tn1 brought in a constitutive promoter firing towards *korC* and *klaABCD*. It is possible that the unregulated transcription from this promoter provided the pressure to protect the *kla* operon from upstream transcription. In the case of the *korABFG* operon the presence of the *kla* operon in RK2 is tightly regulated by KorA and KorB and should not therefore have caused a problem, providing no obvious explanation for the need for a terminator.

Conservation and drift of *korA*

degree to which drift has occurred is quite remarkable (Fig. 3a). There is apparently no identity except in regions which should be functionally conserved, such as the -10 , -35 , O_A and O_B regions, unless a number of gaps are introduced. This suggests that drift has occurred both by base deletion/insertion and by base substitution.

The *korC* genes are also highly conserved. However the least conserved region is the proposed helix–turn–helix motif (Kornacki *et al.*, 1990). Although there are only two KorC-regulated promoters in R751, it is interesting to note that the putative KorC operators are identical to each other but different by two bases from the RK2 KorC operators (Fig. 5). These changes are symmetrical and preserve the perfect inverted-repeat structure. The changed operator sequence may correlate with the changes in the KorC protein. This identity of KorC operators within each plasmid subfamily suggests that there is strong selective pressure to maintain similar repressor–operator interactions at all regulatory points on a single genome. Gene conversion may provide the means to transfer any mutational changes from one control region to another.

It has been proposed that the ancestral IncP plasmid contained an operon consisting of *kbcA* (*kilC*), *kbcB* (*prekorC* or ORFX) and *korC* (Thomas *et al.*, 1988; Larsen & Figurski, 1994). R751 appears to contain this operon intact. The high degree of conservation of the *kbcA* ORF suggests that it still plays an important role in the survival of these plasmids (Fig. 6). It has been noted (Larsen & Figurski, 1994) that KlcA shows 31% sequence identity with the ArdB protein of the IncN plasmid pKM101 (Belogurov *et al.*, 1993), suggesting that KlcA may form part of an antirestriction system which could help RK2 to overcome restriction barriers during establishment after conjugative transfer. Fig. 5 includes the ArdB protein in the alignment of KlcA proteins. The degree of identity with the R751 KlcA (32%) is very similar to that with RK2 KlcA. It is clear that the regions of identity with ArdB lie within the regions of highest identity between the two KlcA proteins, which is consistent with the idea that the sequence conservation represents selection for maintenance of a common structure or function. However, the motif that Belogurov *et al.* (1993) found to be conserved between different antirestriction proteins (ColIb Ard, pKM101 ArdA, pKM101 ArdB and T7 0.3 gene product), LLREYVNTL in ArdB, termed the Ard Box, is not conserved between ArdB and the IncP proteins. Nor is the equivalent region conserved between the IncP proteins themselves.

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Fig. 6. (a) Alignment of KlcB from RK2 and R751. (b) Alignment of domains conserved between KorA, TrbA and KlcB.

identity but diverge to the greatest extent at their N-termini. It is possible that this drift is due to the loss of selection on RK2 *klcB* after its inactivation by the insertion of Tn1. However, the retention of an intact ORF does not support this idea.

A conserved domain in *klcB*

Sequence comparisons revealed a region in the *klcB* ORF preceding *korC* which had strong similarity to the C-terminal domains of KorA and TrbA (Fig. 6; Thomas reported in Pansegrau *et al.*, 1994; Larsen & Figurski, 1994). Our sequence comparisons have shown quite clearly that this region is likely to form a separate domain joined to the DNA-binding domain by a short variable region (G. Jagura-Burdzy & C. M. Thomas, unpublished results). The segment in KlcB covers the whole of this putative 35 amino acid domain. In addition, it may be significant that an insertion of seven amino acids in RK2 which is not present in R751 occurs precisely in the proposed variable region, strengthening the idea that this is a linker region between two domains and so can tolerate considerable modification.

R751 has an extra KorB binding site upstream of *klcA*

The location, organization and spacing of regulatory sequences are almost identical between the two plasmids. KorA operators are located upstream of –35 regions for *kleAp* and *klcAp* and overlapping the –10 for *korAp*. Putative KorC operators overlap –10 regions for *kleAp* and *klcAp*. A KorB operator is found downstream of *korC* in both plasmids, although it is unclear whether binding of KorB in this region plays a role in regulating either *korC* or *kleA*.

The only difference between the two plasmids is in the presence of an extra KorB operator directly upstream of the KorA operator in the *klcA* promoter. We would expect KorB to bind to this site but the spacing between the KorB and KorA operators is much closer here than observed elsewhere. The possibility that binding of these two regulatory proteins is mutually exclusive at these sites should be investigated since this may represent a novel aspect of modulation of gene expression by products of the central control operon.

Conservation of other proteins

The other conserved ORFs encode KleA, KleB, KleE and KleF. The degree of sequence identity varies from 53% (KleB) to 68% (KleA). Comparison to proteins in the databases with known activities have failed to identify likely functions for these gene products. KleA has a high predicted level of α -helical content and a rather regular arrangement of hydrophobic residues interspersed with hydrophilic residues, which could suggest the formation of a coiled-coil structure. However, this feature is less obvious in R751 than it is in RK2. KleB shows a helix–turn–helix motif. This is flanked by four conserved cysteines with a spacing which aligns well with a variety of zinc-finger motifs. These cysteines could coordinate to Zn^{2+} to provide a means of holding the helix–turn–helix motif rigid as an alternative to a larger protein scaffold to which helix–turn–helix motifs are normally attached. Although the gene regulation phenotype, KorE⁺, is associated with this region, the mapping carried out by Kornacki *et al.* (1993) is not consistent with the KleB protein being responsible for this phenotype. Neither KleE nor KleF display features which clearly indicate structure or function. KleG shows about 30% identity with an internal segment of a fusaric acid resistance protein from *Pseudomonas cepacia* as well as low-level similarity to a variety of integrases.

Concluding remarks

The sequence comparisons described in this paper allow us to define more closely the IncP backbone which is conserved between α and β subfamilies. This provides a simpler picture than is presented by the IncP α plasmids alone. Studies on other regions of the plasmids have assigned many of the genes which are co-regulated by the central control operon to specific functions such as replication, transfer and stability (Shingler *et al.*, 1984a, b; Smith *et al.*, 1984; Motallebi-Veshareh *et al.*, 1990, 1992;

Thomson *et al.*, 1993). This left the *korA-oriV* region with the largest number of unassigned genes. The combination of the comparison described here (summarized in Fig. 7), which shows that only the *klc* and *kle* operons belong to the conserved core, and the suggestion by Larsen & Figurski (1994) that the *klc* operon may provide an antirestriction system, reduces the number of apparently redundant genes. We hope that this comparison will aid future work to focus on the most important genes and the conserved motifs within these genes, as well as helping to simplify the picture of these uniquely integrated plasmid genomes.

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