RELAXATION COMPLEXES AND MOBILISATION PROPERTIES OF COLE1 AND RELATED PLASMIDS

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by

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ABSTRACT

In this thesis the role of plasmid relaxation complexes was investigated. A correlation between the levels of mobilisation and relaxation was demonstrated for the wild type ColE1 plasmid. A previously undiscovered cryptic plasmid (pLG500) was found to be present in the ColK$^+$ clinical isolate of E.coli - K216. This plasmid was shown to be a 1 megadalton autonomous replicon, which was non-mobilisable (by R64drd11 and RP4) and non-relaxable. However, in the presence of either of the two closely related plasmids ColK and ColE1, pLG500 was rendered relaxable, and mobilisable by the appropriate sex factors.

Following the comobilisation of pLG500 and ColE1, or pLG500 and ColK, 2-5% of the Col$^+$ transconjugants were found to contain novel plasmids which were about 1 megadalton larger than ColE1 or ColK. Restriction analysis demonstrated that these novel plasmids were formed by site-specific recombination between pLG500 and ColE1 or ColK, and further mating experiments showed that such recombination occurred independently of the recA gene product. These composite plasmids apparently possessed higher copy numbers and produced more colicin than ColE1 or ColK, yet were less relaxable and less efficiently mobilised than the parental Col plasmids. Whilst no evidence for recombination (leading either to the formation or dissociation of these cointegrate plasmids) could be found in vegetatively growing cells, some recipients containing the two original plasmids could be found following the mobilisation of cointegrates.

The unique sites within the ColE1 and ColK plasmids at which recombination with pLG500 occurred were mapped using restriction endonucleases. In the case of ColE1 this site was mapped to within 47 base pairs of the site at which relaxation nicking occurs. For ColK the recombination site was localised to within the same 350 base pair fragment (the HaeII E fragment) as is known to contain a sequence essential for ColK mobilisation. An explanation for this novel recombination phenomenon is presented which strongly suggests that single-stranded DNA transfer from, and strand recircularisation at, the relaxation nicking site, is the underlying process in the mobilisation of ColE1 and related plasmids.

CONTENTS

CHAPTER 1

INTRODUCTION

This project is primarily concerned with the small non-conjugative plasmid ColE1, and other closely related plasmids of Escherichia coli. ColE1 is a 4.2 megadaltons (Mdal), high copy number plasmid, which encodes production of the bacteriocin colicin E1. Unlike larger plasmids, such as F, ColE1 is a non self-transmissible plasmid, however it is often efficiently transferred (mobilised) during sex factor mediated conjugation.

Many plasmids of E.coli and other bacteria can be recovered from host cells as supercoiled DNA bound to proteins which are capable of introducing a unique single-stranded nick into the DNA, so that it is converted to the open circular, or relaxed, form. The aim of this thesis was to ascribe a physiological role to these 'relaxation complexes', and specifically to the ColE1 complex, since in this case both the plasmid and the complex were well characterised.

At the outset of this project the favoured opinion was that relaxation complexes were involved in vegetative replication, but in some cases they might also have a role in plasmid transfer. However, thereon data accumulated which strongly suggested a role for the ColE1 relaxation complex in plasmid mobilisation, and accordingly this project evolved into a study of the mechanism of transfer of ColE1 and related plasmids. Therefore the experiments reported here will be mainly concerned with relaxation complexes and plasmid mobilisation, and the possible role of such complexes in vegetative replication will only be briefly discussed.

I. DISCOVERY OF THE COLE1 RELAXATION COMPLEX

Over ten years ago Bazaral and Helinski (1968) reported the isolation and characterisation of the plasmid ColE1 as a small (4.2 Mdal), supercoiled, double-stranded , circular DNA molecule. They had isolated ColE1 DNA by dye-buoyant density equilibrium gradient centrifugation of whole cell lysates. In this procedure, developed by Radloff, Bauer and Vinograd (1967), any proteins weakly bound to the DNA are removed because of the high salt concentration, and the remaining supercoiled DNA bands at a discrete density in the gradient because less ethidium bromide can intercalate within it than within linear fragments of chromosomal DNA or nicked circular plasmid DNA. The following year, as an approach to studying the physiology of this plasmid, Clewell and Helinski (1969) devised a method for isolating ColE1 molecules in a state which they presumed would more closely resemble the in vivo condition. Cells were harvested and converted to spheroplasts so that they could be lysed using non-ionic detergents, which would not disrupt protein structure, nor destroy weak bonds between proteins and DNA. Then whole cell lysates were subjected to a 'clearing spin' of a high enough g force to preferentially remove most of the chromosomal DNA. A discrete peak consisting of ColE1 molecules, some still bound to cellular material, could then be detected following neutral sucrose gradient velocity sedimientation of such cleared lysates.

Supercoiled ColE1 molecules prepared in this way had a higher sedimentation coefficient (24S) than supercoiled ColE1 DNA from dye-buoyant density gradients (23S), indicating that they were still complexed to cellular material, presumably proteins. Treatment of these DNA-protein complexes with agents known to affect protein structure (such as Pronase, heat, or sodium dodecyl sulphate - SDS) resulted not

simply in removal of the protein part of the complex, but rather in nicking of the supercoiled ColE1 DNA, and therefore conversion of the covalently closed DNA to its open circular, or relaxed, form. This passive relaxation of supercoiling was accompanied by loss of most of the protein component of the complex, but strong (probably covalent) attachment of the remaining protein to the relaxed DNA. Clewell and Helinski (1969) proposed that the supercoiled ColE1 DNA-protein complex which they had isolated harboured a latent endonuclease activity which was fortuitously activated by protein denaturing treatments. The activation could either be due to the removal of a second protein which was a repressor of the enzyme, or it could be the result of preferential denaturation of a region within the endonuclease which allosterically repressed the catalytic activity.

At the time when the ColE1 relaxation complex was discovered it was apparent that nicking of a supercoiled plasmid molecule was a prerequisite for its semi-conservative replication and for segregation of concatenated daughter molecules, yet no gene products able to fulfil such functions had been identified. Thus the endonuclease activity of the ColE1 relaxation complex was considered a prime candidate for an enzyme involved in vegetative DNA replication.

This project was started seven years after the initial isolation of the ColE1 relaxation complex and an acknowledgment of its possible role in ColE1 plasmid DNA replication. By 1976 it was known that many different plasmids could be isolated as relaxation complexes. Furthermore, the ColE1 complex had been extensively characterised, so that its physical structure, the events associated with induced relaxation, and the effects of changing environmental conditions on its production, were relatively well understood. However, despite much descriptive information, a physiological role had not been ascribed to the relaxation

complex of ColE1 or any other plasmid.

In the next section a historical perspective on the characterisation of the ColE1 relaxation complex (until 1976), and the ideas generated about its possible physiological role, is presented. Since it soon became apparent that relaxation complexes were implicated in the process of sex factor DNA transfer, as well as vegetative replication, it is helpful first of all to briefly outline and compare these two modes of plasmid DNA replication.

I. (a) Models for Plasmid DNA Replication

Two different models for replication of circular DNA molecules have been proposed. Originally a distinction between these two types of replication was made on the basis of the different structures of their replication intermediates (θ or σ) when viewed by electron microscopy. The essential features of the models proposed to account for the 'Cairns' and 'Rolling Circle' modes of replication are shown in figure 1.1.

The Cairns mode, which represents the mechanism of vegetative plasmid DNA replication, has been reviewed by Kasamatsu and Vinograd (1974). This process entails non-covalent attachment of nascent DNA to the template strand, and since no DNA polymerases are known which can initiate strand synthesis de novo, RNA priming is apparently a prerequisite for initiation of DNA synthesis at the replication origin. DNA synthesis of the leading (+) strand then occurs in the 5' to 3' direction, whilst complementary (-) strand synthesis (probably involving RNA priming) occurs in the opposite overall direction. Replication can be unidirectional (as shown in figure 1.1) or bidirectional, depending on whether one or both replication forks move relative to the replication origin. Unless a mechanism for redistributing the torque of a supercoiled molecule is postulated (and this would be energetically very unfavourable) then initiation and continuation, as well as

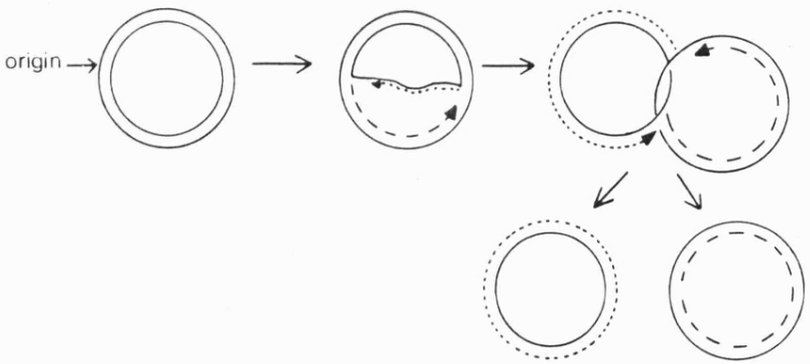
Figure 1.1: MODELS FOR PLASMID DNA REPLICATION

(a) The Cairns Model.

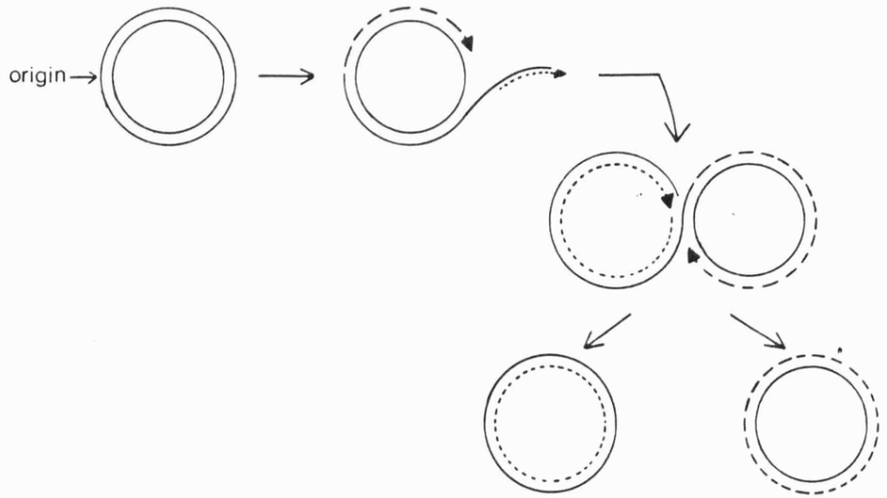
(b) The Rolling Circle Model.

In both cases unidirectional replication is depicted. The arrows indicate the 3' ends of newly synthesised DNA strands. Leading strand synthesis is represented by dashed lines, and lagging strand synthesis by dotted lines.

a) Cairns mode (unidirectional replication)



b) Rolling circle mode



termination, of replication, all require nicking of the DNA. Since plasmid replication intermediates are generally found to be covalently closed, partially supercoiled molecules, this means that ongoing synthesis entails transient nicking and resealing of the DNA (probably in the unreplicated region ahead of the replication fork) to mediate continued strand unwinding.

The Rolling Circle mode of replication has been reviewed by Dressler (1975). This mode represents the mechanism of sex factor DNA transfer, as well as production of viral single strands by ØX174. Here a round of replication is initiated by nicking of the (+) strand at the replication (transfer) origin. The 3' terminus can then be used as a primer for leading strand synthesis without recourse to RNA priming for initiation of replication. Consequently the 5' terminus is displaced from the template, enabling complementary strand synthesis (involving RNA priming) to occur on the displaced single-stranded tail. In contrast to the Cairns model, the newly synthesised (+) strand is covalently attached to the parental DNA, and replication intermediates contain only one replication fork. Furthermore, since the replication fork itself can act as a swivel, it is not necessary to invoke transient nicking and resealing of the DNA to account for strand unwinding during ongoing synthesis. Completion of this process requires DNA cleavage, and recircularisation of the linear molecule which is released.

The principal differences between these two replication processes reside therefore in the initiation and termination steps, whilst ongoing complementary strand synthesis could well involve common enzymes. Finally, it is important to note that some replicons can, under different conditions, use either of these two modes of replication.

II. CHARACTERISATION OF THE COLE1 RELAXATION COMPLEX

II. (a) Strand-specificity of Relaxation Nicking

When DNA of relaxed ColE1 complex was denatured, and the single strands of DNA prepared by sedimentation in alkaline sucrose gradients, Clewell and Helinski (1970a) found that equal proportions of unit length linear and circular strands were recovered. This demonstrated that relaxation of the complex entailed only a single nicking event per molecule. In addition, the circular and linear strands were found to cross, but not self, hybridise, indicating that the relaxation nick was strand-specific. The following year Blair, Clewell, Sherratt and Helinski (1971) showed that the two strands of ColE1 DNA exhibited different buoyant densities when bound to poly UG, and they were therefore able to define the ColE1 strand nicked at relaxation as the one with the greater affinity for poly UG (the poly UG heavy strand).

The strand-specificity of induced relaxation nicking reinforced the view that the relaxation complex did have some role *in vivo*. Relaxation complexes had by then been found for several different plasmids, and in the case of ColE2 (Blair *et al.*, 1971) and F (Kline and Helinski, 1971), where experiments were done to identify which strand was nicked, the site of relaxation was also found to be in the poly UG heavy strand. Blair *et al.* (1971) suggested that the proteins of the relaxation complex produced the initial nick in supercoiled ColE1 DNA which was necessary for strand unwinding during vegetative replication. It seemed reasonable that the initiation of ColE1 replication might involve a strand-specific nicking step, since previously it had been found that the initiation of DNA replication at lambda prophage induction apparently involved strand-specific nicking (Gilbert and Dressler, 1968).

Vapnek and Rupp (1970) had shown that a unique strand of F factor

DNA was always transferred during conjugation, and this was the poly UG heavy strand. Therefore Kline and Helinski (1971) concluded that in the case of F (where the strand nicked at relaxation was also the one transferred during conjugation) relaxation nicking might have a role not only in the initiation of vegetative replication, but also in initiating single-stranded DNA transfer during conjugation.

II. (b) The Site of Relaxation Nicking

In 1974 Lovett, Guiney and Helinski, mapped the site of ColE1 relaxation nicking with reference to the plasmid's single EcoRI restriction site. This was done by determining the sizes of the single-stranded products of EcoRI restriction of relaxed complex. Their results showed that relaxation nicking was site-specific, occurring about 19% of the genome length away from the EcoRI site. In a similar way Lovett, Katz and Helinski (1974) had also shown (by electron microscopy of EcoRI restricted replication intermediates) that ColE1 replication proceeded unidirectionally, from a replication origin/terminus located about 19% of the genome length away from the EcoRI site. Whilst their data did not distinguish whether the relaxation site was the same side of the EcoRI site as the replication origin, they proposed that these sites were in the same vicinity, and that relaxation proteins might have a role in nicking the DNA at the origin/terminus, thus participating in either initiation or termination of vegetative replication. The following year Sugino, Tomizawa and Kakefuda (1975) confirmed that these sites were in fact (at least roughly) coincident.

II. (c) Proteins of the ColE1 Relaxation Complex

The strand and site specificity of relaxation nicking supported the proposal by Clewell and Helinski (1970) that the supercoiled DNA of the relaxation complex was bound to proteins, one of which possessed an endonuclease activity. When relaxation nicking was first reported a

distinction was made between the possibility that it involved activation of an endonuclease which was bound to the DNA, or destruction of a protein bridge which maintained the supercoiling of previously nicked DNA. Studies of the relaxation complexes of ColE2 (Blair et al., 1971), R6K (Kupersztoch-Portnoy, Lovett and Helinski, 1975), and eventually ColE1 (Lovett and Helinski, 1975) showed that it was possible to dissociate relaxation proteins from the supercoiled DNA without inducing relaxation. Therefore, even if enzymic nicking of the DNA and relaxation of supercoiling are not simultaneous, the distinction made by Clewell and Helinski (1970) is only temporal, and so here the relaxation complex will be considered as covalently closed supercoiled DNA bound to relaxation proteins.

The proteins of the ColE1 relaxation complex were characterised in 1975. Complex which had been purified by successive sucrose gradient centrifugation steps, was DNAase treated, and the proteins left were analysed by SDS polyacrylamide gel electrophoresis (SDS-page). In this way Lovett and Helinski (1975) showed that three proteins with molecular weights of 60,000, 16,000 and 11,000 were bound to the supercoiled DNA. Similarly it was shown that the 16,000 and 11,000 daltons proteins were liberated from the complex at relaxation, while a single molecule of the 60,000 daltons protein became strongly bound to the relaxed complex. However, obviously it was impossible to conclude from these experiments which one protein, or combination of proteins, effected relaxation nicking or attachment of the 60,000 daltons protein to the relaxed ColE1 DNA.

Blair and Helinski (1975) showed that the protein with a molecular weight of 60,000 remained associated with relaxed complex even after treatment with phenol, formamide, 3 M urea, or 2 M lithium chloride: such tenacity suggested that it was covalently linked to the DNA. By

preparing denatured, relaxed complex (using alkaline caesium chloride gradients), it was shown that the single strand of the ColE1 complex which was less dense, due to protein binding, was the relaxed strand. The relaxed complex was then used as a substrate for several nucleases and polymerases, to establish whether the 60,000 daltons protein was bound to the poly UG heavy strand at the terminus of the relaxation nick, and if so whether it was at the 3' or 5' end. Guiney and Helinski (1975) showed that relaxed ColE1 complex was a substrate for DNA polymerase I, and this meant it had a free 3'OH terminus. The relaxed complex was not a substrate for T7 exonuclease, indicating that the 5' end was inaccessible. Furthermore, T7 exonuclease could digest relaxed ColE1 DNA after it had been Pronase treated (to completely remove the protein), but not after trypsin disruption of the protein moeity, thus reinforcing the view that the 60,000 daltons protein was attached to the 5' terminus by covalent binding. Since relaxed ColE1 DNA possessed a terminal 3'OH group, Guiney and Helinski suggested that the 60,000 daltons protein was probably bound to the relaxed strand by attachment of an amino acid residue to the 5' phosphoryl group. Such a DNA-protein linkage can potentially conserve the energy of the nicked phosphodiester bond, and could therefore facilitate ligase-type closure of nicked DNA.

When the conformation of replicating ColE1 molecules was examined (Lovett et al., 1974), it was found that unreplicated regions remained supercoiled, implying that parental strands remained covalently closed for most of the time during replication. Therefore Guiney and Helinski (1975) suggested that transient nicking and resealing of replicating ColE1 DNA, necessary for strand unwinding during ongoing replication, could be mediated by the 60,000 daltons protein of the relaxation complex. In the case of F, which was known to transfer its own DNA, the

authors postulated that relaxation nicking initiated conjugal DNA transfer, and a relaxation protein bound to the 5' (leading) end could facilitate ligation of the transferred single strand, to yield a covalently closed circular molecule, in the recipient. Since recombination of transferred strands occurred in recA as well as Rec^+ recipients, indicating that it did not simply involve reciprocal recombination between homologous sequences, it had previously been difficult to envisage how this process was mediated. Therefore the proposals of Guiney and Helinski (1975) were conceptually very important, as they underlined the possibility of a simple and elegant mechanism of strand recircularisation which relied upon protein-DNA, rather than DNA-DNA, interactions.

In summary, the physical characterisation of the ColE1 relaxation complex showed that it consisted of supercoiled DNA bound to three major protein components, with molecular weights of 60,000, 16,000 and 11,000. As shown in figure 1.2, induced relaxation entails nicking of the DNA at a unique site (which was at that time indistinguishable from the vegetative origin), and in a unique strand (the poly UG heavy strand). Relaxation nicking, which is accompanied by passive unwinding of supercoiling, results in covalent attachment of the single molecule of the 60,000 daltons protein to the 5' nick terminus, and liberation of the 16,000 and 11,000 daltons protein components.

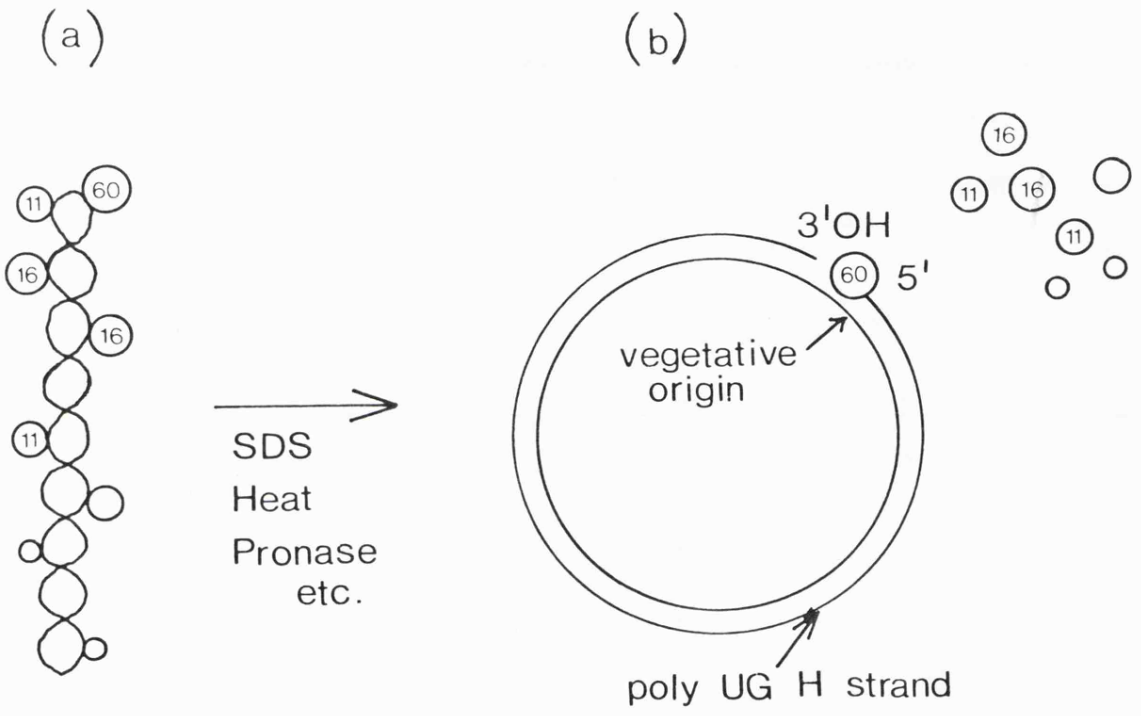
II. (d) Factors Affecting Levels of Relaxation

Clewell and Helinski (1972) found that 30% of the ColE1 molecules isolated from cells grown in the presence of glucose were SDS-relaxable, whereas of those grown in the absence of a carbon source (or plus glycerol), 80% were relaxable. This difference in the proportions of complexed molecules was independent of cell growth rate, but was to some extent strain dependent. Later Katz, Kingsbury and Helinski (1973),

Figure 1.2: INDUCED RELAXATION NICKING OF THE COLE1 RELAXATION COMPLEX

(a) The ColE1 relaxation complex consists of a supercoiled plasmid DNA molecule non-covalently complexed to relaxation proteins of 11K, 16K, and 60K, as well as other minor unidentified protein species.

(b) Induced relaxation nicking of the complex results in the covalent attachment of the 60K protein to the 5' terminus of the nicked (poly UG heavy) strand, and dissociation of the other proteins. Nicking occurs at a unique site indistinguishable from the replication origin.


(a)
(b)
11
60
16
16
11
SDS
Heat
Pronase
etc.
16
11
16
11
3'OH
60
5'
vegetative
origin
poly UG H strand

showed that this glucose effect on the production of relaxation proteins was due to catabolite repression. They showed that the addition of cyclic adenosine 3', 5'-monophosphate (c-AMP) stimulated production of relaxation proteins even when plasmid DNA synthesis was inhibited. (Normally c-AMP treatment causes a transient increase in ColE1 DNA synthesis, as well as an increase in the proportion of molecules present as relaxation complexes.) The authors also reported that different c-AMP levels did not alter the efficiency with which ColE1 was mobilised from donor cells during R64drd11 mediated conjugation, suggesting that the proportion of molecules complexed did not affect the efficiency of ColE1 mobilisation.

Clewell (1972) reported that during inhibition of cellular protein synthesis by chloramphenicol treatment ColE1 DNA continued to replicate semi-conservatively, even though no new rounds of chromosomal DNA synthesis could be initiated. Whilst the extent of such replication varied according to the medium in which cells were grown, in general during the second to fourth generation equivalents the rate of ColE1 replication was significantly greater than under normal circumstances, and thereafter the plasmid DNA continued to replicate at a constant rate for 10-15 hours, when cells might contain several thousand copies of the plasmid.

Since ColE1 continued to replicate in the absence of protein synthesis, it was possible to follow the fate of preformed relaxation proteins. Clewell and Helinski (1972) found that during chloramphenicol treatment, as ColE1 molecules accumulated, the actual number of complexed molecules did not decrease. This indicated that once they had been produced, relaxation proteins were stable for at least several generations. Similar proportions of ColE1 molecules which had replicated before and after inhibition of protein synthesis were found to be

complexed, and this implied that the relaxation proteins could associate reversibly, and at random, with different ColE1 molecules in a cell. Bazaral and Helinski (1970) had shown, by density shift experiments, that during a cell generation the probability that a molecule would be duplicated appeared to be independent of whether or not it had already been used as a replication template. (These experiments were performed in Rec^+ strains, so the apparent independence might simply be explained by reciprocal recombination between once-replicated ColE1 molecules.) The implied random association of relaxation proteins was compatible with the proposal that relaxation nicking was an essential step in vegetative replication, yet equally it was compatible with the hypothesis that the relaxation complex represented the repressed state with regard to replication. If relaxation nicking was necessary for replication, these experiments demonstrated that relatively few sets of relaxation proteins could efficiently participate in replicating many ColE1 molecules. 80% of the ColE1 molecules isolated from cells grown in the presence of high c-AMP levels were SDS-relaxable, yet by inhibiting protein synthesis Clewell and Helinski (1972) were able to demonstrate that there was an excess of relaxation proteins, sufficient to saturate three times this amount of DNA. One interpretation, again compatible with the proposal that the relaxation complex was the repressed state with regard to replication, was that the minor, non-complexed, portion of ColE1 molecules were newly replicated ones which had to mature (i.e., be modified) before they could complex with relaxation proteins.

Finally, the observation made by Clewell (1972), that the rate of ColE1 replication transiently increased during chloramphenicol treatment, was compatible with the proposal that relaxation proteins exerted a negative control over ColE1 replication. If bound relaxation

proteins acted as repressors of ColE1 replication (for example, by preventing initiation of a round of DNA synthesis), then during chloramphenicol treatment, as the proportion of complexed molecules fell, runaway synthesis of ColE1 DNA should result. Such synthesis would continue until some other factor, such as availability of replication enzymes, became rate-limiting. However, if inhibition of chromosomal DNA synthesis results in the liberation of replication enzymes which are normally associated with the chromosome, then an alternative explanation of the transient increase in ColE1 replication rate could be that this plasmid uses the excess of replication enzymes which are made available.

When the ColE1 relaxation complex was first described (Clewell and Helinski, 1969), it was shown that different treatments caused a different proportion of the complexed molecules to relax. This means that some treatments which induce relaxation nicking underestimate the number of complexed molecules in a sample. Similarly, some plasmids (for example, the small mobilisable plasmid described by Humphreys, Grindley and Anderson, 1972) cannot be isolated as a relaxation complex. In these cases it may be that the relaxation complex does not exist in vivo; alternatively, it may be that the equivalent of a relaxation complex does exist, but because of its fragility the proteins of the complex dissociate even during the gentle lysis procedure developed for isolating the ColE1 relaxation complex.

Relaxation complexes of different plasmids may not respond to all the same treatments. Clewell and Helinski (1970b) found that whilst heat treatment induces relaxation of the ColE1 complex, it inactivates ColE2 and ColE3 relaxation complexes. Since ColE2 and ColE3 plasmids are not related to ColE1 in terms of DNA sequence (Inselburg, 1973; Warren and Sherratt, 1977), this suggests either that there are common

host-coded relaxation proteins which interact differently with different plasmids, or, more likely, that at least some relaxation proteins are plasmid-coded.

II. (e) Other DNA-Protein Complexes

By 1976 many different plasmids of E.coli and other bacteria, had been isolated as relaxation complexes. As shown in table 1.1, wherever studied, relaxation nicking was found to be site and strand specific. These general features of the relaxation process reinforced the idea that there was a common physiological role for different plasmid relaxation complexes.

Not all plasmid DNA-protein complexes which are isolated consist solely of relaxation proteins bound to DNA. If ColE1 is isolated by lysis with a non-ionic detergent, but prepared by neutral sucrose sedimentation of the cleared lysate in the presence of 0.05 M, rather than 0.5 M, sodium chloride (Clewell and Helinski, 1970a), it sediments as a larger (34S) complex. The additional cellular material which is bound at low ionic strength can be removed by 0.5 M sodium chloride, yielding the 24S relaxation complex. Furthermore, Lovett and Helinski (1975) found that although the 60,000, 16,000 and 11,000 dalton proteins were the major components of the relaxation complex, there were several other proteins bound to the DNA in minor amounts, but these were preferentially lost during successive purifications of the complex by neutral sucrose gradient centrifugation.

The plasmid CloDF13, from Enterobacter cloacae, has been isolated as a protein-DNA complex (Veltkamp, Blankevoort and Nijkamp, 1975). However, treatment with ionic detergents dissociates the complex, so it cannot be considered a relaxation complex. The complex contains one protein with a molecular weight of 60,000, and this has been identified as the cloacin DF13 molecule. This bacteriocin apparently binds to a

Table 1.1

RELAXATION COMPLEXES OF SEVERAL CONJUGATIVE AND NON-CONJUGATIVE PLASMIDS

Plasmid	Mol. Wt. (Mdal)	Conjugative	Site-Specificity of Relaxation	Strand-Specificity of Relaxation	References
ColE1	4.2	No	At or near origin of replication	Poly UG H	Blair et al. (1971) Lovett et al. (1974)
ColE2	5.0	No	Yes	Poly UG H	"
ColE3	5.0	No	Not tested	Not tested	Clewell and Helinski (1970b)
R6K	25	Yes	At or near origin of replication	Poly UG H	Kupersztoch-Portnoy et al. (1975)
F	62	Yes	Not tested	Poly UG H	Kline and Helinski (1971)
ColIb	62	Yes	Not tested	Not tested	Clewell and Helinski (1970c)

specific region of the CloDF13 DNA, but does not interact with other DNA species. In the presence of Mg^{++}, cloacin converts the supercoiled DNA to a linear form which is capable of recircularisation (indicating that the DNA has staggered breaks). Whether this complex has any physiological significance is not known.

Finally, viral DNA-protein complexes with features similar to plasmid relaxation complexes have also been found. In vitro studies of ØX174 have shown that production of circular viral strand progeny from the double-stranded, supercoiled, replicative form (which entails rolling circle replication), is initiated by endonuclease nicking by the cisA protein, which is bound to ØX174 DNA at the replication origin. Nicking at the origin is accompanied by covalent attachment of the cisA protein to the 5' end, and during replication the 5' terminus backtracks along the displaced viral strand, and the cisA protein nicks again at the regenerated replication origin. The cisA protein then completes the production of a circular viral molecule by ligating the new 3'OH end and the 5' complexed end of the resultant linear molecule (Eisenberg, Griffith and Kornberg, 1977). A DNA-protein complex has also been described for SV40 (Kasamatsu and Wu, 1976). When SV40 is isolated in the presence of SDS and dithiothreitol, the open circular fraction of the DNA contains a protein species which is covalently bound to the nick terminus. In the case of SV40 the nick can be in either strand of the molecule, but is located near the origin of replication and transcription.

III. PROSPECTS FOR EVALUATING THE ROLE OF RELAXATION COMPLEXES

By 1976 Helinski and his co-workers, who were virtually the only workers in this area, had put forward two main proposals for the possible roles of plasmid relaxation complexes. Firstly relaxation complexes were implicated in the process of vegetative replication, where relaxation nicking could have a role in the initiation, continuation, or termination of replication, or where the relaxation complex could represent the repressed state with regard to DNA duplication. Secondly it was thought that relaxation complexes of conjugative plasmids could participate in DNA transfer, as well as in vegetative replication. Relaxation nicking could create the origin of transfer, and a relaxation protein bound to the 5' terminus of the relaxed strand could mediate its transfer, and its recircularisation in the recipient.

At this time it was apparent that the transfer of small plasmids, such as ColE1, was dependent upon sex factor mediated conjugation. The possibility that transfer of ColE1 molecules entailed anything other than their passive diffusion into the recipient had not been considered. Therefore a common role for relaxation proteins, of both conjugative and non-conjugative plasmids, in the process of DNA transfer, was never proposed; instead it was assumed that the ColE1 relaxation complex must be involved in vegetative replication.

When F relaxation was found to entail a single nicking event, specifically in the strand transferred during conjugation (Kline and Helinski, 1971; Vapnek and Rupp, 1970), this led to the proposal that relaxation complexes of conjugative plasmids might be involved in initiating conjugal DNA transfer, as well as in some aspect of vegetative replication. However, even when the ColE1 complex was more extensively characterised, and it became clear that all its

properties were compatible with a specialised role for ColE1 relaxation proteins in single-stranded DNA transfer, these findings did not provoke an investigation of whether ColE1 played some part in its own transfer. Instead, this possibility was overlooked, and the properties of the ColE1 relaxation complex were used to rationalise how conjugal transfer of F DNA might occur. Later (for example, see Inselburg, 1977a; Dougan and Sherratt, 1977) it was to become clear that ColE1 did play some part in its own transfer. At the time this was a very surprising finding. However, if it had been known earlier, then the idea that relaxation complexes must be involved in vegetative replication might not have been so often stated and widely accepted. Indeed, an appraisal of the known properties of the ColE1 relaxation complex in view of this finding certainly favours the hypothesis that relaxation proteins have a role in ColE1 transfer, whereas the data neither supports nor refutes a role for such proteins in ColE1 DNA duplication.

Despite the subjectivity of their approach, Helinski and his co-workers provided an excellent basis for studying the function of the ColE1 relaxation complex. They had compiled a detailed description of the physical properties of the ColE1 complex, and, where possible, demonstrated similarities to other relaxation complexes. Such detailed knowledge, and the accompanying increase in more general information, would prove invaluable in relating structure and function of the complex.

At the outset of this project two general strategies which could be used to identify a role for relaxation complexes were considered. Clewell and Helinski (1972) had shown that it was possible to alter the proportion of ColE1 molecules which were complexed by chemically altering the environment. One approach to identifying a function for relaxation complexes would be to attempt to correlate

changing levels of complex with some physiological change. In fact, Helinski's group had used this approach, but had found that under conditions where there should be significantly different levels of complex, replication was apparently normal, and the mobilisation frequency of ColE1 was unaltered. However, as I will discuss later, the second of these experiments was not rigorous, and therefore does not fairly represent the value of this approach. A second approach would be to isolate mutants of ColE1 with altered relaxation characteristics, and to attempt to identify a common physiological difference or defect. Intrinsically this approach is considerably more powerful, but is initially more time-consuming since one or more series of derivatives have first to be characterised. Working alone, and within a time limit, a decision was made to combine these approaches, first of all studying wild-type ColE1 under different conditions, and then if it seemed relevant, making use of the mutant derivatives of ColE1 which were available.

IV. STUDIES OF COLE1 MOBILISATION

By 1977 three different groups had isolated and characterised series of ColE1 derivatives made by insertion of an ampicillin resistant transposon. Since insertion of a transposon within a gene inactivates the gene function, by mapping sites of insertion and examining changes in phenotype it was possible to relate ColE1 structure and function. In this way So, Gill and Falkow (1975) identified several different mutants which were defective in colicin production. Later, in more extensive studies, Inselburg (1977a), and Dougan and Sherratt (1977), were able to map mutations affecting colicin immunity, ColE1 incompatibility, and relaxation complex levels, in addition to those disrupting colicin production.

The isolation of ColE1 derivatives which were only poorly relaxable demonstrated that at least one component of the relaxation complex was plasmid-coded. Inselburg (1977a) reported that all of the relaxation deficient mutants he had isolated apparently replicated and segregated their DNA normally. Previously Clewell (1972) had shown that wild-type ColE1 continued to replicate when levels of relaxation complex were very low (during prolonged chloramphenicol treatment). Both these findings corroborated the view that if relaxation proteins had a role in ColE1 plasmid DNA duplication it was likely to be at a moderating level.

Inselburg (1977b), and Dougan and Sherratt (1977), compared the transfer efficiency of ColE1 derivatives with normal and lowered levels of relaxation complex. They both found that there was a rough correlation between the proportion of molecules complexed and the efficiency of mobilisation of the different derivatives from Hfr or R64drd11 donors. Whilst Inselburg found that some mobilisation deficient derivatives had no relaxation complex, Dougan and Sherratt found that all their

mobilisation deficient mutants had significant levels of complex, even though these levels were lower than those of mobilisation proficient derivatives. One reason for this difference, and for discrepancies within a series (ranked in order of mobility), may be that it is difficult to accurately measure the proportion of supercoiled and open-circular DNA by sucrose gradient analysis.

Finding mutants of ColE1 which were defective in mobilisation was both very surprising and important. It showed that ColE1 did play some part in its own transfer, a possibility which had not been considered before, probably because of the known dependence of ColE1 transfer on sex factor mediated conjugation. Furthermore, when a map of ColE1 was compiled which showed the extent of transposon insertions affecting plasmid mobilisation (Dougan, Saul, Warren and Sherratt, 1978), it became obvious that a substantial part of the genome was involved in ColE1 mobilisation. In fact a single region spanning about one quarter of the ColE1 genome was found to be essential for mobilisation. At first it might seem bizarre that a small non-conjugative plasmid should code any transfer functions at all. However in the absence of selective advantage plasmids are eventually lost from a bacterial population. It may be that in nature ColE1 often coexists with a sex factor which can promote its mobilisation. Under such circumstances the ability of ColE1 to be mobilised would be important both for its continued survival and for its dissemination to different bacterial hosts.

As discussed previously, once it became clear that ColE1 played some part in its own transfer, the known properties of its relaxation complex were obviously compatible with the hypothesis that the relaxation proteins were involved in the process of ColE1 mobilisation. The finding that mobilisation deficient derivatives were in general

less relaxable than wild type ColE1 strongly, if indirectly, supported this hypothesis.

In the following sections of this chapter the present day state of knowledge of the ColE1 mobilisation process will be reviewed. All the experiments that I am about to describe were reported whilst this project was in progress. Together they provided good circumstantial evidence for a role for relaxation proteins in ColE1 transfer. Since the original aim of this project was to investigate the physiological role of relaxation complexes, these findings obviously influenced the aims and choices of experiments I will be describing in subsequent chapters.

IV. (a) Complementation of the ColE1 Mobility Defect

Dougan and Sherratt (1977) had isolated ColE1 derivatives by mobilising ColE1 from a strain carrying either R64drd11 or F_{ts} trp lac to promote conjugation, and RP4 as the donor of the ampicillin resistance transposon, Tn1. Since it seemed likely that mobilisation deficient ColE1::Tn1 derivatives would not be recovered by this method, chloramphenicol amplified plasmid DNA was also prepared from these strains, and used to transform cells to ampicillin resistance. However, Dougan and Sherratt found that one of the ColE1::Tn1 derivatives obtained by the original procedure could not be mobilised in subsequent crosses. This observation prompted a detailed investigation of the possibility that ColE1 could complement the transfer defect of these Mob^- mutants (Warren and Sherratt, 1977), and a less extensive analysis of this phenomenon was also made by Inselburg (1977b).

Experiments involving the comobilisation of wild-type ColE1 and mobilisation deficient Tn1 derivatives were difficult to perform because ColE1 cannot stably coexist with very closely related plasmids. Following the construction of appropriate donor strains, then unless dual selection could be applied, one or other plasmid would cease to replicate and would be diluted out of the population at subsequent cell divisions. Although the proportion of donor cells carrying both plasmids at the time of mating was perforce very low, the results of these experiments clearly demonstrated that the presence of wild-type ColE1 compensated for the mobility defect of these ColE1::Tn1 derivatives. Since this occurred in recA donors, and plasmids were unaltered after transfer to the recipients, Warren and Sherratt excluded the possibility that transfer of Mob^- mutants simply entailed reciprocal recombination with the largely homologous wild-type ColE1 plasmid, and consequent transfer as a single linkage group. They also showed that ColK, which is closely related to, but

compatible with, ColE1, could complement the mobility defect, whereas the unrelated plasmid ColE2 could not. Warren and Sherratt concluded that ColE1 coded for trans-acting gene products which were essential for mobilisation; furthermore, ColK coded its own mobilisation proteins, and these were similar enough to those of ColE1 to function instead of them.

Warren and Sherratt proposed that wild-type ColE1, or ColK, plasmids were able to complement the transfer defect of Mob$^-$ ColE1 derivatives because ColE1 possessed a specific transfer origin with which mobilisation proteins, at least some of which were plasmid-coded, interacted in trans to initiate DNA transfer. Similarly Inselburg (1977b) concluded that ColE1 coded for mobilisation proteins which acted in trans. He suggested that since mobilisation deficient mutants of ColE1 were also defective in relaxation complex, some mobilisation proteins might be components of this complex, and one or more of them might, by effecting site-specific relaxation nicking, be responsible for creating the origin of single-stranded ColE1 DNA transfer.

These experiments marked the beginning of an analysis of ColE1 mobility functions. They were also of practical importance because they showed that some apparently non-mobilisable ColE1 derivatives, which were therefore considered relatively safe cloning vectors (for example, see Armstrong, Hershfield and Helinski, 1977), could actually be quite efficiently transferred in the presence of related wild-type plasmids.

IV. (b) The ColE1 Relaxation Site and the ColE1 Transfer Origin

Within the limits of resolution afforded by electron microscopic measurements of plasmid DNA, it had been shown that the site of ColE1 relaxation nicking and its origin/terminus of replication were coincident (Lovett, Guiney and Helinski, 1974; Sugino et al., 1975). Similarly, the relaxation site and origin of bidirectional replication of the conjugative

plasmid R6K were found to be coincident (Lovett, Sparks and Helinski, 1975). These findings did much to further the view that relaxation complexes were involved in the process of plasmid DNA vegetative replication.

In 1977 Tomizawa, Ohmiri and Bird, determined the exact location of the ColE1 replication origin using DNA sequencing techniques. On the basis of electron microscopic evidence, ColE1 DNA replication was known to proceed from the same site, and in the same direction, *in vitro* as *in vivo* (Tomizawa, Sakakibara and Kakefuda, 1974; Lovett, Katz and Helinski, 1974). Furthermore, during *in vitro* DNA synthesis it was possible to rescue (from early replication intermediates) the initial DNA fragment made; this nascent DNA species was a 6S L strand fragment, comprising about 400 nucleotides (Sakakibara and Tomizawa, 1974). By sequencing the region previously shown to contain the replication origin, and then locating within it the 5' end of the nascent 6S fragment, Tomizawa *et al.* (1977) were able to precisely define the location of the ColE1 replication origin. As shown in figure 1.3, a unique feature of the origin region is the cluster of five AT base pairs, preceded one turn of the helix away by an array of nine GC base pairs. This is a characteristic arrangement for a termination signal of RNA polymerase activity, resulting in dissociation of the transcription complex (Roberts, 1976). Bastia (1977) also sequenced a region of approximately 260 base pairs surrounding the ColE1 replication origin, and his data confirmed the presence of the origin sequence shown in figure 1.3. The following year Bastia (1978) also mapped the site of ColE1 relaxation nicking. On the basis of the increased molecular weight due to covalent attachment of a protein to the relaxation nick terminus, it was possible to identify which restriction fragment of relaxed ColE1 complex contained the site of relaxation nicking. In this way he showed that the ColE1 relaxation site was physically

distinct from the origin of vegetative replication. The sequence surrounding this site is shown in figure 1.3; the salient feature is its two-fold rotational symmetry, which might be indicative of a protein recognition site.

The discovery that the ColE1 replication origin and relaxation site were physically distinct, and separated by about 270 nucleotides (Tomizawa et al., 1977; Bastia, 1978), demanded a modification of the ideas implicating relaxation complexes in vegetative replication. It was still possible to propose ways in which relaxation complexes might participate in vegetative replication, but these proposals were now justified only by the proximity, not coincidence, of the two sites.

In order to localise a ColE1 transfer origin Warren, Twigg and Sherratt (1978) generated HaeII deletion mutants of a mobilisation proficient ColE1::Tn1 plasmid, and by examining the mobility phenotype of the derivatives, showed that a non-complementable mobility defect resulted specifically from the loss of the HaeII C fragment. This restriction fragment was already known to contain the ColE1 relaxation site, but not the replication origin (Tomizawa et al., 1977; Bastia, 1978). These experiments showed that the HaeII C fragment also contained the ColE1 transfer origin, or basis of mobility (bom), site (Warren et al., 1978). Because of the correlation between mobility and relaxability, Warren et al. proposed that the site within the HaeII C fragment which was indispensable for transfer was the relaxation site. By cloning a region of about 300 base pairs spanning the relaxation site into a plasmid which was apparently unrelated to ColE1 and not mobilised in its presence, they were able to show that the recombinant plasmid was both mobilisable and relaxable in the presence of ColE1.

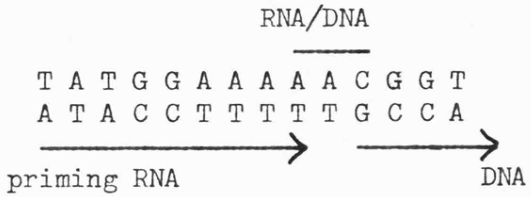
These experiments did not prove that the ColE1 relaxation site was the transfer origin, nor that relaxation complexes were involved in transfer. It was after all a predictable result that a recombinant plasmid

Figure 1.3: DNA SEQUENCE SURROUNDING THE COLE1 REPLICATION ORIGIN AND THE RELAXATION NICKING SITE

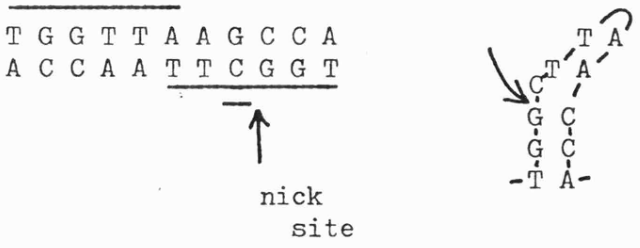
(a) The replication origin, showing the priming RNA/DNA junction.

(b) The relaxation site, showing the nicking site and the two-fold rotational symmetry in this region.

(a) Replication origin



(b) Relaxation site



carrying the cloned ColE1 relaxation site should become relaxable when ColE1 relaxation proteins were provided in *trans* (for example, see Clewell and Helinski, 1972). However, these experiments did show that the ColE1 transfer origin was at least quite close to the relaxation site. Moreover, the features of the ColE1 relaxation event were compatible with relaxation proteins having a central role in the process of plasmid DNA transfer via a rolling circle type of model. Therefore these experiments strongly suggested that the ColE1 relaxation site was the transfer origin, and that relaxation nicking was a prerequisite for ColE1 DNA transfer.

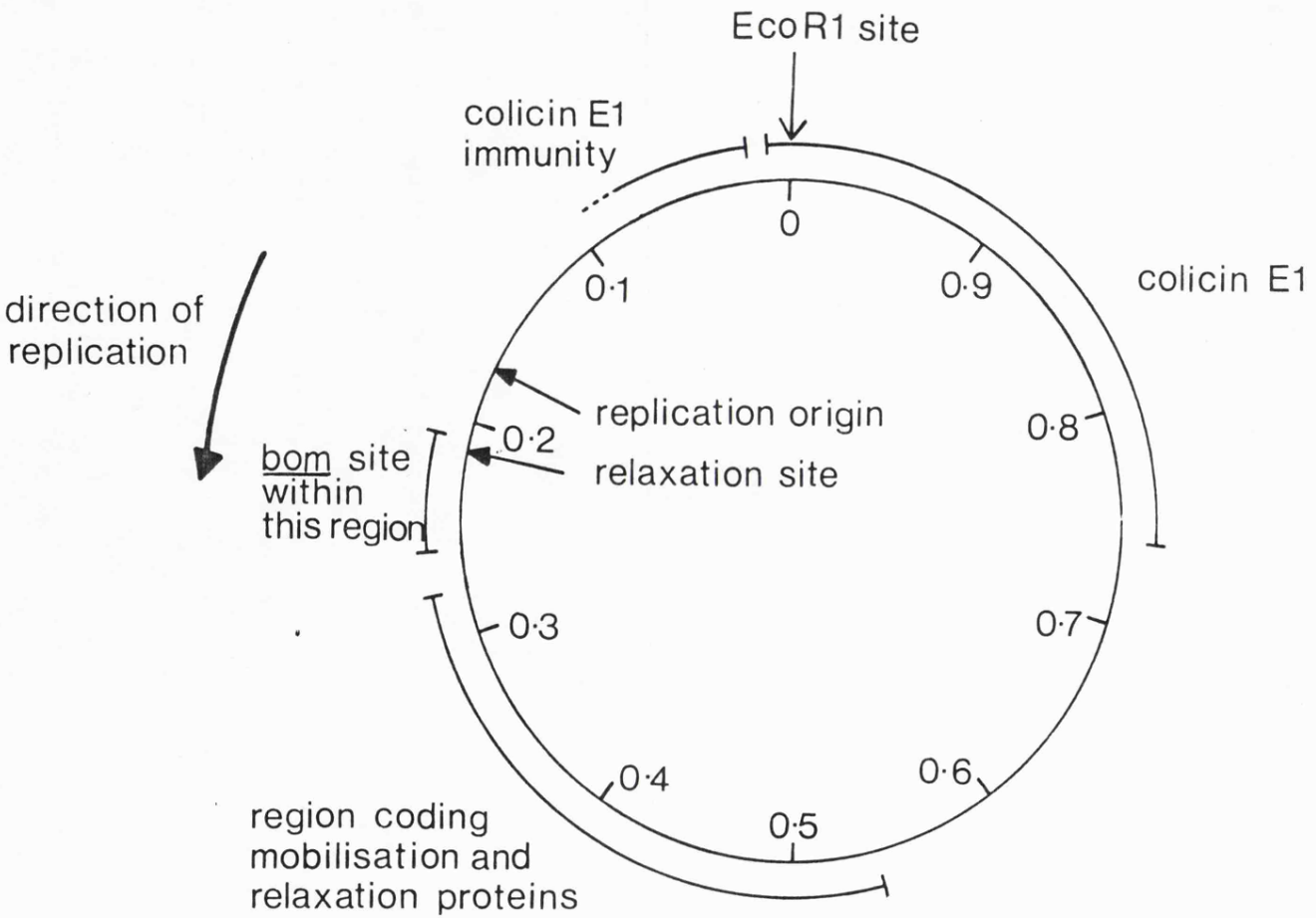
Two incidental, but important, conclusions from these manipulations were that the presence of the ColE1 relaxation site was not essential for plasmid replication and maintenance, and that the proteins of the relaxation complex can bind to the plasmid DNA in the immediate vicinity of the relaxation nicking site.

IV. (c) Gene Products involved in ColE1 Mobilisation

Mobilisation studies of ColE1 and its derivatives enabled the localisation of a single region (with ColE1 map coordinates 0.28-0.54), and a *cis*-active site (within coordinates 0.21-0.26), which were both essential for mobilisation (see figure 1.4). Since mutations within the region 0.28-0.54 resulted in a Mob^- phenotype which could be complemented by wild-type ColE1, this suggested that this region coded diffusible gene products (mobilisation proteins) which interacted with the *bom* site to initiate, and possibly mediate, mobilisation. Proof that this coding region and the *bom* site were all that was necessary for mobilisation was later obtained by cloning them into an unrelated plasmid which was non-mobilisable, and showing that it could then be mobilised during F or R64*drd*11 mediated conjugation (Warren, Saul and Sherratt, 1979).

Since complementation of the ColE1 mobility defect was accompanied

Figure 1.4: A FUNCTIONAL MAP OF PLASMID COLE1


EcoR1 site
colicin E1 immunity
0
0·1
0·9
colicin E1
direction of replication
replication origin
0·2
relaxation site
0·8
bom site within this region
0·3
0·7
0·4
0·6
0·5
region coding mobilisation and relaxation proteins

by increased relaxability, and the site essential for mobilisation was at least very close to the relaxation site, it seemed likely that one or more mobility proteins were components of the relaxation complex. Attempts to identify mobility proteins, and correlate these with previously characterised relaxation complex proteins, were therefore made. This was done by comparing the patterns of polypeptide production by mobilisable and non-mobilisable derivatives of ColE1, both in minicells (Dougan and Sherratt, 1977; Inselburg and Applebaum, 1978), and during in vitro protein synthesis (Collins, 1979).

Both Dougan and Sherratt (1977), and Inselburg and Applebaum (1978), found that the Mob$^-$, relaxation deficient, phenotype correlated with the absence of a small polypeptide, and they reported its molecular weight as 15,000, and 16,000, respectively. They concluded that this protein might be the 16,000 daltons protein which had been previously identified as a component of the ColE1 relaxation complex (Lovett and Helinski, 1975). However, the patterns of polypeptide synthesis of ColE1 obtained in these two studies were quite different, so it is not clear whether the same protein had been identified in both cases. The results obtained by Inselburg and Applebaum can perhaps be considered uncharacteristic, since those of Dougan and Sherratt do (for ColE1) agree fairly well with another similar study (Meagher, Tait, Betlach and Boyer, 1977). When the products of in vitro protein synthesis were examined (Collins, 1979), three new proteins, two with a molecular weight of 28,000 and one of 3,000, whose absence correlated with the Mob$^-$ phenotype, were identified. A 15,000 daltons protein was also implicated, but since it was present in very small amounts, it was probably a breakdown product of one of the 28,000 daltons proteins. Just as results of different minicell studies had been at variance, the results of Collins were at least quantitatively different from those of similar studies (for example, see Yang and Zubay, 1978).

In summary, no convincing correlations between ColE1-coded mobility proteins and relaxation proteins, could be drawn from these studies. This was not surprising firstly because although production of certain polypeptides could be correlated with the mobility phenotype, whether these were representative of the proteins produced *in vivo* was not known. Moreover, there was no evidence that all of the proteins of the relaxation complex were plasmid-coded, and some ColE1-coded mobility proteins could be involved in forming the complex, without themselves becoming components of it.

Another approach to identifying ColE1-coded mobility proteins is that of Inselburg and Ware (1979), who have performed a complementation analysis of mutants within the mobility region 0.28-0.54, and thus established that there are three complementation groups spanning it. Whilst it is impossible to ascribe any one of these genes to the production of a specific protein, it certainly seems likely that the 60,000 daltons protein is too big to be coded in this region (unless there are overlapping genes). This protein, which is a candidate for the site-specific endonuclease (since it is recovered bound to the nicked DNA after relaxation), may therefore be a host-coded gene product.

In order to perform the complementation analysis of the mobility region, Inselburg and Ware isolated Mob^- mutants of a mobilisation proficient ColE1 derivative, following hydroxylamine treatment of the plasmid DNA *in vitro*. Surprisingly, the majority of Mob^-, putative point mutants were still relaxable. In view of the wealth of observations implicating relaxation proteins in mobilisation, and since point mutants can have much less drastic effects on protein function than insertions or deletions, this result implied that relaxation proteins have another role in mobilisation after relaxation nicking has occurred.

It is known that F plasmid DNA transfer entails a rolling circle type of mechanism. Evidence for the transfer of a specific (poly UG heavy) strand of F DNA, by its leading 5' terminus, was obtained by prelabelling the DNA of donor cells before mating, and then characterising the labelled products which could be recovered from recipients after conjugation (Rupp and Ihler, 1968; Vapnek and Rupp, 1970). Similar experiments have demonstrated that the transfer of some I-like and F-like R plasmids also occurs in this way (Vapnek, Lipman and Rupp, 1971). However, such experiments have not been reported for ColE1, probably because not enough ColE1 DNA is transferred to enable the detection of labelled ColE1 DNA in the recipients. The molecular mechanism of ColE1 DNA transfer is therefore entirely speculative. However, an attractive hypothesis, on the basis of the possible coincidence of the ColE1 transfer origin and the relaxation nicking site, is that relaxation proteins mediate ColE1 DNA transfer by a process which closely resembles that of sex factor DNA transfer. The role of relaxation proteins would be to nick (in the poly UG heavy strand) at the relaxation site, thus creating the origin of single-stranded DNA transfer. The relaxation protein covalently bound to the 5'end could then participate in transferring this strand, and in effecting its recA independent recircularisation in the recipient. A specific model of this type, but based on analogy with ØX174 rolling circle replication, has been proposed by Warren, Twigg and Sherratt (1978). They have proposed that the 60,000 daltons protein of the relaxation complex is functionally similar to the cisA protein of ØX174 (see Eisenberg et al., 1977). According to their model the 60,000 daltons protein binds to the leading 5' end of the relaxed strand, and during single-stranded DNA transfer it backtracks the incoming strand, nicks again at the regenerated transfer origin (relaxation site), and religates the

transferred 3' and 5' termini to yield a recircularised plasmid molecule in the recipient.

It is important to stress that there is no evidence that relaxation nicking and covalent attachment of a relaxation protein to the 5' nick terminus occurs *in vivo*. Nor is there evidence that the ColE1 relaxation site actually corresponds to the *bom* site. The finding made by Inselburg and Ware (1979), that some mobilisation defective mutants are still relaxable, is certainly compatible with the hypothesis that relaxation proteins mediate transfer and/or recircularisation of transferred strands, but obviously it does not provide proof of this hypothesis. In conclusion, whilst it has been shown that some ColE1-coded proteins are necessary for mobilisation, the actual role of these proteins in the process of ColE1 DNA transfer is not known.

The gene products involved in sex factor DNA transfer are firstly those encoding production of the pili and other cell surface structures which provide the 'machinery' for DNA transfer, and secondly those involved in processing (i.e., replicating) the DNA during transfer. Obviously, all the gene products involved in fulfilling the structural requirements for sex factor DNA transfer are also necessary for plasmid mobilisation. However, even when all the structural requirements have been met, in the case of some sex factors, for example R100.1 (unpublished observations), ColE1 is not mobilised at all. Since ColE1 can be isolated as a relaxation complex regardless of whether a sex factor is also present, and since this complex is strongly implicated in the mobilisation process, it may be that F and R64*drd*11 (but not R100.1) promote the mobilisation of ColE1 because they code gene products which trigger the activity of the ColE1-coded mobility proteins, specifically during conjugation, for example resulting in relaxation nicking by the proteins of the ColE1 relaxation complex.

Whilst the incompatibility P group plasmid R751, and the incompatibility W group plasmid R388, both promoted the mobilisation of wild-type ColE1, it was found that they did not promote the mobilisation of the chimaeric plasmid containing only the sequences of ColE1 which were essential for F or R64drd11 promoted mobilisation (Warren, Saul and Sherratt, 1979). A reassessment of the mobilisability of different ColE1::Tn1 derivatives by these sex factors demonstrated that additional regions of the ColE1 genome were essential for mobilisation. These findings implied either that the process of ColE1 mobilisation was quite different when promoted by R751 and R388 than by F or R64drd11, or that the same basic process was always involved but some sex factors could not provide all the gene products necessary for triggering mobilisation, and this deficiency was sometimes complemented by extra ColE1-coded gene products. These findings were very unexpected, for they showed that the process of ColE1 mobilisation was more complex, and involved more of the ColE1 genome, than had previously been suspected. In summary, either there were multiple mechanisms for ColE1 mobilisation, or multiple interactions (rather than just the provision of sex factor coded gene products) were sometimes necessary to initiate transfer.

As yet no sex factor coded gene products which are specifically involved in promoting mobilisation have been identified. It is interesting that R100.1 does not promote the mobilisation of ColE1, for this plasmid is closely related to F, to the extent that at least six transfer genes common to both plasmids have been identified (Ohtsubo, Nishimura and Hirota, 1970). Several mutants of F which are defective in conjugal transfer have been shown to still promote the mobilisation of the small plasmid CloDF13 (Van de Pol, Veltkamp and Nijkamp, 1978), and it has been known for some years that a traI mutant of F, which is defective in the DNA nicking step that initiates transfer, still

promotes ColE1 mobilisation (Reeves and Willetts, 1974). However, in view of the findings made by Warren et al. (1979), it is possible that even if any of these mutants were defective in promoting mobilisation, their deficiency would be complemented by the conditional mobility proteins coded by the small plasmid, and therefore remain undetected.

V. COLE1 RELAXATION PROTEINS AND VEGETATIVE REPLICATION

Once it became clear that ColE1 played an active part in its own transfer, a common role for relaxation complexes of conjugative and non-conjugative plasmids in the process of plasmid DNA transfer could be envisaged, and it was no longer necessary to propose that ColE1 relaxation proteins must be involved in the process of vegetative replication. However, it has recently been shown that the temperature-sensitive replication phenotype of one mutant of ColE1 correlates with an alteration in the properties of its relaxation complex (Collins, Yanofsky and Helinski, 1978), and several past observations are at least compatible with a role for relaxation proteins in the control of ColE1 replication. Therefore, in this section I will try to clarify the possible involvement of ColE1 relaxation proteins in the process of vegetative replication.

In 1975 Tomizawa, Sakakibara and Kakefuda found that *in vitro* replication of ColE1 occurred in cell free extracts of *E.coli* prepared from a strain which had not carried the ColE1 plasmid. Recently the replication of ColE1 in the absence of any plasmid-specified proteins has also been demonstrated *in vivo*, using ColE1 plasmid-phage hybrid molecules (Donoghue and Sharp, 1978; Kahn and Helinski, 1978). Therefore relaxation proteins are not essential for ColE1 vegetative replication, and this implies that if they have a role it is at a moderating level.

It is known that very closely related plasmids cannot be stably maintained in the same cell. The phenomenon of plasmid incompatibility was first reported by Scaife and Gross (1962), who found that an F'lac plasmid could not be stably maintained in an Hfr strain, and it has since been used to group naturally occurring plasmids according to their relatedness (Datta and Hedges, 1971). It is well documented that different ColE1

derivatives are incompatible, and, in general, for any two derivatives the higher copy number (smaller) plasmid will continue to replicate and be maintained, whilst the lower copy number plasmid ceases replication and is diluted out of the bacterial population at subsequent cell divisions (for example, see Warren and Sherratt, 1978). A popular model to account for plasmid copy number control is the repressor dilution theory, proposed by Pritchard, Barth and Collins (1969). According to this model, a plasmid-coded trans-acting repressor molecule inhibits replication by binding at or near the plasmid replication origin. As cell growth dilutes out the repressor, a critical concentration is reached at which replication can occur. Since synthesis of repressor is coupled to DNA replication, initiation results in the production of more repressor, thus reducing the possibility of further rounds of replication occurring. Clearly, plasmid incompatibility would occur as the consequence of this, or any other, process which results in copy number control. In turn, the study of plasmid mutants with altered incompatibility properties may yield information about the components and mechanism of copy number control.

Recently Hashimoto-Gotoh and Inselburg (1979a; 1979b) have identified a region of ColE1 which is essential for the expression of ColE1 incompatibility. Following the construction of a hybrid plasmid, consisting of a ColE1::Tn3 derivative linked via its single EcoRI site to a pSC101 temperature-sensitive replication mutant, it was possible to isolate deletion mutants which lacked the ColE1 replication origin. Unlike the original hybrid plasmid, some of these no longer expressed incompatibility with a ColE1::Tn5 derivative, and by mapping the extent of deletions, a region essential for the expression of ColE1 incompatibility was identified. This region is about 500 bases long, and is located on the opposite side of the replication origin to

the relaxation site (starting at base 187). The expression of ColE1 incompatibility does not require provision of any ColE1-coded relaxation proteins or the presence of the relaxation site. Moreover, an RNA transcript has been identified which is coded in the incompatibility region, and it is tempting to speculate that this RNA species acts in *trans* as a repressor of replication.

Although ColE1 relaxation proteins are not needed for vegetative replication of ColE1, and do not appear to have a role in ColE1 copy number control, Collins *et al.* (1978) have isolated a ColE1 mutant in which the altered components of the relaxation complex clearly have an effect on replication. This mutant of ColE1 is temperature-sensitive for replication and is also more sensitive to heat-induced relaxation nicking than wild-type ColE1. In 4 out of 5 cases where reversion to a temperature-resistant phenotype occurred, this was accompanied by partial reversion to temperature-resistant relaxation nicking. The ColE1 relaxation site is located 270 bases away from the replication origin, in the pathway of replication. The initial DNA species made during *in vitro* replication of ColE1 is a fragment of about 400 nucleotides, synthesised using the poly UG heavy strand as the template. Relaxation nicking, which occurs in this strand, might disrupt the production of this early replication intermediate, and consequently halt any further replication. Similarly, an alteration in the binding properties of the relaxation proteins might prevent either the initiation of replication, or the completion of this early intermediate. By virtue of the position of the relaxation site, mutations which affect the configuration or activity of the relaxation complex may therefore fortuitously affect replication. Moreover, since relaxation nicking apparently has the specific role in the ColE1 mobilisation process of creating the ColE1 transfer origin, then in the wild-type plasmid (where nicking presumably

only occurs during conjugation) it may have the additional role of preventing any further vegetative replication, thus triggering the switch from a Cairns to a Rolling Circle mode of replication.

CHAPTER 2

MATERIALS AND METHODS

I. BACTERIAL STRAINS AND PLASMIDS

The bacterial strains used are described in table 2.1. For AB2463, GB15, GB2, and their derivatives, the *recA* phenotype was confirmed by subjecting cells to a uv dose of 300 ergs/mm^2. This dose is sufficient to kill *recA* strains, whereas *recA*$^+$ strains survive.

The plasmids used are listed in table 2.2. Recipients of R64*drd*11 and RP4 were selected with 25 µg/ml tetracycline; pML21 was selected with 25 µg/ml kanamycin sulphate. Counterselection against donor strains was made using 150 µg/ml streptomycin sulphate (Glaxo), 100 µg/ml spectinomycin hydrochloride (Upjohn), or 100 µg/ml naladixic acid.

II. MEDIA

In general Luria broth, containing 10 g tryptone, 5 g yeast extract, and 5 g NaCl per litre, adjusted to pH 7.0, was used for growing cells in liquid culture. Occasionally nutrient broth, containing 25 g Oxoid number 2 nutrient broth per litre, was used.

When experiments involved radioactive labelling of the DNA, cells were grown in M9 minimal salts medium containing per litre: 1 g NH_4Cl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 11 mg $CaCl_2$, and 246 mg $MgSO_4.7H_2O$. This was supplemented with 0.5% casamino acids, and 0.2% (w/v) glucose or 0.2% (v/v) glycerol as the carbon source.

Nutrient agar plates contained 25 g Oxoid number 2 nutrient broth and 14.5 g Davis agar per litre. Antibiotics were added at the concentrations indicated in the previous section.

Table 2.1

BACTERIAL STRAINS

Strain	Characteristics	Source
K216	colicin K producing isolate of *E.coli*	P. Fredericq
K235	colicin K producing isolate of *E.coli*	P. Fredericq
AB2463	*thr leu thi pro his argE rpsL lac gal ara xyl mtl recA13*	
MC5	*thr leu thi deoB deoC thyA*	P. Meacock
GB2	*his trp lys gal lac rpsL* Su^- ^{R} $T6^R$ colicin I^R	G. Buttolph
JB1	*thr leu thi lacY rpsL* colicin $E1^R$ colicin K^R	derived from C600
JB2	*thr leu thi lacY nalA*	derived from C600
5K	*thr leu thi hsdR tonA supE*	W. Brammar
GB15	*thi (lac pro)* XIII *rpsE recA*	G. Buttolph

Table 2.2

BACTERIAL PLASMIDS

Plasmid	Characteristics	Source
R100.1	F-like Sm^r Tc^r	B.M. Wilkins
R64drd11	I-like Sm^r Tc^r	B.M. Wilkins
RP4	Inc-P Tc^r Km^r Ap^r	N.J. Grinter
ColE1-K30	wild type ColE1	D.R. Helinski
ColE2	wild type ColE2	D.R. Helinski
ColK	wild type ColK from K216	P. Fredericq
pLG500	cryptic miniplasmid from K216	P. Fredericq
pML21	mini-ColE1-Km^r	P. Meacock
ColE1-Ap	ColE1-Ap^r	R. Diaz

III. PREPARATION OF SUPERCOILED PLASMID DNA

400 ml Luria broth cultures were grown overnight to stationary phase. Cells were harvested by centrifugation in a Sorvall GSA rotor at 8,000 r.p.m. (10,000 g) for 10 minutes at 10°C. They were resuspended in 3-5 ml of 25% (w/v) sucrose dissolved in 50 mM tris(hydroxymethyl)aminomethane, adjusted to pH 8.0 with HCl (tris-HCl, pH 8.0). Cells were converted to spheroplasts by adding 1 mg lysozyme per ml, and, after 5 minutes, 10 µg diaminoethanetetra-acetic acid, disodium salt (EDTA) per ml. This mixture was left for 5 minutes, and the spheroplasts were then lysed by adding 2% (v/v) Triton X-100, dropwise with gentle swirling. In general a final concentration of 0.2% Triton X-100 was sufficient for cell lysis. Whole cell lysates were cleared by centrifugation in a Sorvall SS34 rotor at 18,000 r.p.m. (40,000 g) for 10 minutes at 10°C. 3.4 ml of the cleared lysate (the supernatant of the clearing spin) was mixed with 6 ml saturated caesium chloride (CsCl) solution, and then 0.6 ml of a 5 mg/ml solution of ethidium bromide (EtBr) was added. 10 ml CsCl-EtBr gradients were centrifuged in a Beckman type 50 Ti rotor at 33,000 r.p.m. (70,000 g) for 36-48 hours at 15°C.

The more dense band of supercoiled plasmid DNA was collected either by dropwise fractionation from the bottom of the tube, or by inserting a syringe needle into the side of the tube and withdrawing the DNA band. This sample was diluted three-fold in TE buffer (1 mM EDTA, 10 mM tris-HCl, pH 7.5). Two volumes of ice-cold absolute ethanol were then added, and the sample was stored at -20°C for 4-15 hours. The supercoiled plasmid DNA was pelletted by centrifugation in a Sorvall HB4 rotor at 12,000 r.p.m. (23,000 g) for 15 minutes at -10°C. Purified plasmid DNA was stored at -20°C in TE buffer.

If strains carried more than one small plasmid, 100 ml cultures, in M9 minimal salts medium supplemented with glucose and casamino acids,

were also set up, and the DNA of exponentially growing cultures was labelled by adding 100 µCi of ^{3}H thymidine (specific activity, 50 Ci/mMol). Cleared lysates were prepared as before, and the labelled and unlabelled samples mixed prior to centrifugation. Following CsCl-EtBr gradient centrifugation, the different supercoiled plasmid DNA species were separated by preparative neutral sucrose gradient centrifugation, as described in section VII.

IV. AGAROSE GEL ELECTROPHORESIS

Agarose slab gels were made by dissolving agarose in electrophoresis buffer consisting of 40mM tris, and 10 mM EDTA, adjusted to pH 7.7 with glacial acetic acid. 5-8 mm thick gels were run submerged in electrophoresis buffer in a horizontal gel apparatus. DNA samples were mixed with one fifth volume of loading buffer containing 1 mg/ml bromophenol blue in 50% (w/v) sucrose. 5-50 µl samples were then loaded into the slots and electrophoresed towards the positive electrode. Usually gels were run for 2-3 hours at 100 volts. If samples did not contain SDS, EtBr, at a final concentration of 0.5 µg/ml, was included in the electrophoresis buffer. If samples contained SDS gels were run in the absence of EtBr, and following electrophoresis they were stained in electrophoresis buffer containing 5 µg/ml EtBr, before being photographed. Gels were photographed on a Spectrolight Transilluminator (Birchover), emitting short wavelength ultraviolet light, using a Nikon camera fitted with an orange filter.

V. DIGESTION OF PLASMID DNA WITH RESTRICTION ENDONUCLEASES

Samples of plasmid DNA (0.5-1 µg in 5-25 µl TE buffer) were digested with 1-2 units of restriction endonucleases for 1-2 hours, at 37°C. In the case of the enzyme TaqYI, the incubation temperature was 50°C instead of 37°C. Prior to the addition of enzyme, DNA samples, in the appropriate

reaction buffers, were incubated at 65°C for 5 minutes, to inactivate any nucleases. The reaction buffer used for HaeII, HaeIII, and HhaI digestion contained 50 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 0.5 mM dithiothreitol. The HpaII reaction buffer contained 20 mM tris-HCl (pH 7.5), 7 mM $MgCl_2$, and 1 mM dithiothreitol; the TaqYI reaction buffer contained 6 mM tris-HCl (pH 7.5), 6 mM $MgCl_2$, 0.5 mM dithiothreitol, and 50 mM NaCl. Restriction endonuclease digestion products were separated by agarose gel electrophoresis in 2-4% agarose gels, made in electrophoresis buffer containing EtBr.

VI. CONTROLLED DEOXYRIBONUCLEASE (DNAase I) NICKING

0.1-0.5 µg of plasmid DNA in 5-20 µl of 10 mM tris-HCl (pH 7.5) and 5 mM $MgCl_2$, was mixed with 0.02 ng of DNAase I (Worthington). Reaction mixtures were incubated for 0-10 minutes at 37°C, and the reaction was then terminated by the addition of excess EDTA. The enzyme was added from a stock solution containing 10 ng DNAase I and 100 ng bovine serum albumin in 1 ml of 10 mM tris-HCl (pH 7.5).

VII. ASSAY OF RELAXATION COMPLEX LEVELS BY NEUTRAL SUCROSE GRADIENT CENTRIFUGATION

A 100 ml culture of the plasmid carrying strain was grown in M9 minimal salts medium supplemented with 0.5% casamino acids, and either 0.2% glucose or 0.2% glycerol as the carbon source. The DNA of exponentially growing cells was labelled by the addition of 50 µCi ^3H thymidine, and cells were harvested in mid log phase. Triton X-100 cleared lysates were made as described in section III, and then diluted in TE buffer to a final sucrose concentration of less than 5%. Two 400 µl samples, one of which had been mixed with SDS (to a final concentration of 0.5%) to induce relaxation nicking, were centrifuged in 5 ml 5-20%

(w/v) sucrose gradients. 5 and 20% sucrose solutions were made in 0.5 M NaCl, 5 mM EDTA, and 50 mM tris-HCl (pH 8.0). Gradients were run in a Beckman SW50.1 rotor at 50,000 r.p.m. (230,000 g) for 2½ hours at 15°C, and equal volume fractions (of 60-120 µl) were collected dropwise from the bottom of the tube onto one inch squares of Whatmans number 1 filter paper. The papers were dried, washed three times in ice-cold 5% trichloracetic acid (TCA) for 10 minutes, twice in ice-cold industrial methylated spirits (IMS) for 5 minutes, rinsed once in acetone, and finally dried under an infra-red lamp. The papers were transferred to plastic scintillation vials, and 0.5 ml of a non-aqueous scintillation fluid, containing per litre of toluene: 33 mg of 1, 4-BIS-2-(4-methyl-5-phenyloxazolyl) benzene (dimethyl POPOP), and 5 g of 2, 5-diphenyloxazole (PPO), was added to each vial. The plastic vials were stoppered, placed in Packard glass scintillation vials, and the samples counted in a Packard Liquid Scintillation Spectrophotometer. Estimates of relaxation complex levels were made by comparing the number of counts in supercoiled and open circular peaks, before and after SDS-induced relaxation.

VIII. EXAMINATION OF THE PLASMID CONTENT OF CELLS BY AGAROSE GEL ELECTROPHORESIS OF SDS CLEARED LYSATES

Cells were grown overnight in 5 ml Luria broth, and sedimented in a Sorvall SM24 rotor. Bacteria were resuspended in 300 µl of 25% (w/v) sucrose, in 10 mM EDTA and 50 mM tris-HCl (pH 8.0). 5 µl of 10% (w/v) SDS was added, and this usually caused rapid lysis of the cells. Whole cell lysates were cleared by centrifugation in an SM24 rotor at 18,000 r.p.m. (40,000 g) for 10 minutes at 10°C, and the supernatants (the SDS cleared lysates) were stored in 1.5 ml microfuge tubes at -20°C.

An alternative method for making SDS cleared lysates was to streak cells across a nutrient agar plate, and, after overnight incubation, resuspend and lyse the bacteria as before, either in SM24 tubes or in 1.5 ml

microfuge tubes. In the latter case, whole cell lysates were vortexed for 10 seconds, and then cleared by centrifugation for 12 minutes in an Eppendorf centrifuge (number 5412).

SDS cleared lysates were mixed with one-fifth volumes of loading buffer (see section IV), and 30-60 µl samples were subjected to agarose gel electrophoresis. Since samples contained SDS, gels were run in the absence of EtBr and then stained after electrophoresis. In general plasmids which were larger than 2-3 Mdal were examined by 0.5% agarose gel electrophoresis. For plasmids of less than 2 Mdal, SDS cleared lysates were treated to remove cellular RNA, and then examined by 1.2% agarose gel electrophoresis. The RNA was removed by adding ribonuclease A (RNAase), at a final concentration of 50 µg/ml, and incubating the SDS cleared lysate for 10 minutes at 37°C. Following RNAase treatment protease was added, at a final concentration of 100 µg/ml, and the sample incubated for a further 10 minutes at 37°C. Protease treatment removes any RNAase bound to the DNA. Before use stock solutions of RNAase and protease (10 mg/ml) were heated to 90°C for 10 minutes, to inactivate any contaminating nucleases.

IX. MOBILISATION EXPERIMENTS

Cells were grown overnight in Luria broth, diluted 1/50, and grown for 4-6 generations (with at least one more dilution step), to a density of approximately 2×10^8 cells per ml. 0.3 ml of the donor strain was added to 3 ml of the recipient, and the mating mixture was incubated, with very gentle shaking, for 30 minutes at 37°C. Conjugation was then terminated by vortexing the mating mixture (in a 25 ml test-tube), at maximum speed, for 30 seconds. Sex factor recipients were obtained by plating dilutions of the mating mixture onto nutrient agar plates containing the appropriate antibiotics. Plasmid mobilisation frequencies,

defined as the % of sex factor recipients also acquiring the small plasmid, were determined either by direct examination of the plasmid content of cells (by agarose gel electrophoresis of SDS cleared lysates), or by scoring R plasmid transconjugants for the appropriate phenotype (such as colicin production or immunity).

X. COLICIN PRODUCTION AND IMMUNITY

Colonies were tested for colicin production by streaking them onto nutrient agar plates overlaid with soft nutrient agar which had been seeded with 10^7-10^8 cells of a colicin-sensitive strain. Following overnight incubation of the plates, colicinogenic cells could be identified by the zone of killing immediately surrounding them, in the bacterial lawn.

Colicin E1, Colicin E2, and Colicin K were prepared by growing colicin-producing cells overnight in nutrient broth, diluting the cultures 1/200, and, when a density of 3-5 x 10^8 cells per ml had been attained, adding mitomycin C to a final concentration of 0.5 ug/ml. Cultures were incubated for a further 5-7 hours at 37°C, and then the cells were sedimented and discarded. The crude colicin preparation (the supernatant) was sterilised by chloroform treatment, and stored at 4°C.

Cells were tested for colicin immunity by cross-streaking against the crude colicin preparation (0.05 ml), on a nutrient agar plate. Following overnight incubation of plates, sensitive strains could be distinguished by the zone of killing where the colicin had been streaked. For colicin-insensitive cells, a distinction between colicin immunity and colicin resistance was made by cross-streaking strains against a second colicin. Colicin resistant mutants were obtained by cross-streaking the appropriate sensitive strain against the colicin preparation, and purifying any colonies growing in the killing zone.

CHAPTER 3

MOBILISATION AND RELAXATION PROPERTIES OF COLE1 AND COLK

I. Introduction

In 1977 Dougan and Sherratt, and Inselburg, reported the isolation of ColE1 mutants which had lowered levels of relaxation complex, and they showed that there was a rough correlation between the levels of relaxation complex and mobilisation efficiencies of these derivatives. Previously however, Katz *et al.* (1973) had found that the mobilisation efficiency of wild type ColE1 was the same regardless of whether cells were grown under conditions which should stimulate production of relaxation proteins. Although ColE1 was incompatible with its Mob$^-$ derivatives, Inselburg (1977b), and Warren and Sherratt (1977), were able to construct transiently heterozygous donor cells and demonstrate that wild type ColE1 could complement the transfer defect of the Mob$^-$ derivatives. Moreover, Warren and Sherratt showed that the plasmid ColK, which is closely related to ColE1 but compatible with it, was also able to complement the mobility defect.

The properties of the ColE1 relaxation complex deduced from *in vitro* studies were compatible with the hypothesis that relaxation proteins had a specialised role in the process of ColE1 plasmid DNA transfer, and specifically that relaxation nicking created the origin for single-stranded DNA transfer. Whilst a physical approach to the study of ColE1 transfer was probably impossible, this hypothesis could be examined indirectly, first of all by determining whether there was a correlation between relaxation complex levels and mobilisability for wild type ColE1, and secondly by seeing whether complementation of the transfer defect of Mob$^-$ ColE1 derivatives was accompanied by

complementation of the relaxation deficiency. The experiments described in this chapter deal with these two questions.

II. Mobilisation and Relaxation Properties of wild type ColE1

Whilst Katz et al. (1973) had reported that wild type ColE1 was mobilised at a similar frequency from donor cells with high or low levels of relaxation proteins, no data was presented to substantiate this statement. Therefore I compared the efficiency of mobilisation of wild type ColE1 from donor cells which had been grown with either glucose or glycerol as the carbon source, and also measured the proportion of ColE1 molecules present as relaxation complex at the time of mating. A short mating time (30 minutes) was chosen for these, and all other, mobilisation experiments, in order to minimise the possibility of multiple rounds of plasmid transfer occurring.

Initially the donor strain MC5/R100.1 + ColE1 was constructed, and this was mated with the colicin E1 resistant recipient JB1. (A colicin E1 resistant recipient was used to prevent killing of recipient cells during mating, by colicin E1 produced by donor cells). However, it was found that R100.1 did not promote the mobilisation of ColE1 at all. The donor strain MC5/R64drd11 + ColE1 was then constructed. Donor and recipient (JB1) strains were grown in M9 minimal salts medium supplemented with 0.5% casamino acids and either 0.2% glucose or 0.2% glycerol. At a density of 2×10^8 cells per ml, the DNA of the donor cultures was labelled by adding ^3H thymidine, and 30 minutes later the glucose or glycerol-grown donor and recipient strains were mated, and the remainder of the donor cultures harvested, and used to prepare Triton X-100 cleared lysates. Aliquots of the cleared lysates, with or without the prior addition of 0.5% SDS, were subjected to analytical neutral sucrose gradient centrifugation (as described in

chapter 2, section VII), in order to assay relaxation complex levels. After the mating mixture had been incubated for 30 minutes mating was terminated (by vortexing the mixture) and R64drd11^{+} recipients were selected by plating on nutrient agar containing streptomycin and tetracycline. R plasmid transconjugants were then scored for colicin production, in order to determine the efficiency of mobilisation of the ColE1 plasmid. The results of this experiment are summarised in table 3.1. In contrast to the report made by Katz *et al.*, this data clearly shows that there is a correlation between the proportion of ColE1 molecules present as relaxation complex, and the efficiency of mobilisation of the ColE1 plasmid.

III. Complementation of the ColE1 Mobility Defect by ColK

Since it seemed likely that different ColE1 relaxation proteins were able to associate with different ColE1 molecules *in vivo* (see Clewell and Helinski, 1972), complementation of the mobility defect of Mob^{-} ColE1 derivatives by wild type ColE1 might be expected to be accompanied by complementation of the relaxation deficiency, regardless of whether the relaxation proteins were actually involved in the plasmid mobilisation process. Therefore, rather than devising ways to examine the ability of wild type ColE1 to complement the relaxation deficiency of its Mob^{-} derivatives, it was considered necessary to see whether a variety of different plasmids could be found that were able to complement the mobility defect, and to then determine whether these would also be able to complement the relaxation deficiency. Since Warren and Sherratt (1977) had reported that ColK could complement the ColE1 mobility defect, the experiments described in this section were performed in order to confirm and extend their observations, and finally to determine whether ColK-mediated complementation of the ColE1

Table 3.1

RELAXATION AND MOBILISATION OF WILD TYPE COLE1 FROM CELLS GROWN USING EITHER GLUCOSE OR GLYCEROL AS THE CARBON SOURCE

Donor and recipient strains, grown in the presence of either 0.2% glucose or 0.2% glycerol, were mated as described in chapter 2. Recipients of R64drd11 were then selected, and 200 R^+ transconjugants from each cross were tested for colicin production. Triton X-100 cleared lysates were made from the donor cultures, and the levels of SDS-inducible relaxation nicking assayed by neutral sucrose gradient centrifugation, as described in chapter 2.

Donor (MC5/R64drd11 + ColE1)	Efficiency of Mobilisation	% Relaxation
+ GLUCOSE	60	33
+ GLYCEROL	87	81

mobility defect was accompanied by complementation of the relaxation deficiency.

Isogenic recA (GB15) donor strains were constructed which carried R64drd11, and ColK, pML21, or ColK and pML21. The source of ColK was the clinical isolate of E.coli, K216. The characterisation of the plasmid content of K216 will be discussed in detail in chapter 4. pML21 is a kanamycin resistant derivative of the mini-ColE1 plasmid pVH51 (see Hershfield, Boyer, Chow, and Helinski, 1976). It is a mobilisation deficient plasmid which contains nearly half of the ColE1 genome, extending from the EcoRI site and including the replication origin and relaxation nicking site, but lacking part of the coding region essential for mobilisation. A recA donor strain was used in order to exclude the possibility of generalised recombination occurring between the two closely related plasmids, ColK and pML21. If such recombination occurred in the donor it would enable pML21 to be passively transferred to recipients as part of the same linkage group as ColK. The recipient used in these mating experiments was the Rec^+, colicin K resistant, strain - JB1. The donor and recipient strains were grown in M9 minimal medium supplemented with 0.5% casamino acids and either 0.2% glucose or 0.2% glycerol, and then mated at a density of 2-3 x 10^8 cells per ml. R plasmid transconjugants were selected, and the mobilisation efficiencies of ColK and pML21 plasmids were determined as the % of R^+ transconjugants which were colicin-producing, and kanamycin resistant, respectively. In the cross GB15/R64drd11 + pML21 x JB1, kanamycin resistant recipients were selected directly from the mating mixture, because they occurred only at very low frequencies.

The results of these mating experiments are summarised in table 3.2. Clearly, they demonstrate that the presence of ColK in donor cells leads to a very large increase in the frequency of transfer

Table 3.2

COLK-MEDIATED COMPLEMENTATION OF THE pML21 MOBILITY DEFECT IN DONORS GROWN USING GLUCOSE OR GLYCEROL AS THE CARBON SOURCE

Isogenic recA donor strains and recipients grown in the presence of either 0.2% glucose or 0.2% glycerol were mated, R64drd11^{+} recipients selected, and 200-400 of these were tested for colicin production and kanamycin resistance. In the case of the donor strain GB15/R64drd11 + pML21, kanamycin resistant recipients were directly selected from the mating mixture.

Donor (GB15)	E.o.m. in the presence of 0.2% glucose	E.o.m. in the presence of 0.2% glycerol
+ R64drd11 + pML21	4.1×10^{-4}	3.5×10^{-4}
+ R64drd11 + ColK	50-60	80-95
+ R64drd11 + ColK	50	90
+ pML21	10	40

of kanamycin resistance from pML21$^+$ donors. An examination of the plasmid content of these Col$^+$, kanamycin resistant, recipients (by 0.5% agarose gel electrophoresis of SDS cleared lysates) shows that they contain both of the parental small plasmids. This observation strongly supports the hypothesis that mobilisation of pML21 is due to the provision of ColK-coded gene products, rather than a consequence of the two small plasmids recombining in the donor. When several kanamycin resistant recipients of the cross GB15/R64drd11 + pML21 x JB1 were examined the parental pML21 plasmid could not be seen. Presumably therefore pML21 is a completely non-mobilisable plasmid, and under these conditions transfer of the kanamycin resistance phenotype occurs as a result of rare transposition events.

The results presented in table 3.2 also demonstrate that there is a glucose-glycerol effect on the efficiency of mobilisation of ColK, and the efficiencies of mobilisation found for this plasmid are very similar to those observed for ColE1 (see table 3.1). This glucose-glycerol effect is also reflected in the extent to which pML21 is complemented for transfer by ColK.

IV. Relaxability of ColK and pML21

In order to determine whether ColK complemented the relaxation deficiency of pML21, the relaxability of both small plasmids isolated from the isogenic donor strains GB15/R64drd11 + ColK, GB15/R64drd11 + pML21, and GB15/R64drd11 + ColK + pML21, was compared. In a preliminary experiment cultures of these strains, grown in glucose minimal medium, were used to prepare Triton X-100 cleared lysates, and the extent of SDS-inducible relaxation was measured. For both pML21 and ColK less than 8% of the supercoiled material was converted to the open circular form by this treatment, and similarly there was no

stimulation of pML21 relaxability in the presence of ColK. Since in a similar experiment ColE1 was found to be 30% relaxable (see table 3.1), whilst pML21 had previously been reported to be relaxation deficient, these results suggested that ColK did not exist in the form of a relaxation complex.

V. Discussion

The results presented in this chapter demonstrate that there is a correlation between the levels of mobilisation and relaxation of wild type ColE1. Moreover, they show that ColK is mobilised at similar frequencies to ColE1 (from glucose and glycerol grown donors), and that it can complement the pML21 mobility defect. Since the mobilisation of pML21 occurs much more efficiently from a glycerol grown ($ColK^+$) donor than from a glucose grown donor, yet ColK is mobilised at similar frequencies regardless of whether pML21 is also present, this shows that ColK codes excess mobilisation proteins (which can be used by pML21) when glycerol is used as the carbon source.

Although the glucose-glycerol effect on the production of ColK mobilisation proteins resembles the cyclic AMP effect on the production of ColE1 relaxation proteins, in a preliminary experiment I was not able to isolate ColK as a relaxation complex. When Warren and Sherratt (1977) found that ColK complemented the mobility defect of pML21 they did not discuss the possibility that ColK-mediated complementation of the pML21 mobility defect might be due to *trans* acting ColK relaxation proteins, even though a rough correlation between the levels of mobilisation and relaxation of ColE1 derivatives had already been established (for example, see Dougan and Sherratt, 1977). In view of my results a likely explanation for this omission was that they had also found ColK to be a non-relaxable plasmid, and so were

unable to demonstrate ColK-mediated complementation of the pML21 relaxation deficiency. No further attempts were made at this time to isolate ColK as a relaxation complex, since, as I will describe in chapter 5, it was possible to demonstrate the trans activity of ColK-coded relaxation protein equivalents by an alternative method.

CHAPTER 4

DISCOVERY AND CHARACTERISATION OF A CRYPTIC MINIPLASMID FROM THE COLICIN K PRODUCING ISOLATE OF E.COLI K216

I. Introduction

In the mobilisation experiments described in the previous chapter the source of ColK was the clinical isolate of E.coli, K216. This strain was used because it apparently contained only the ColK plasmid. However, the experiments I shall describe here revealed that K216 contained a very small plasmid in addition to ColK, and that this miniplasmid was transferred from K216 during R64drd11 mediated conjugation. The discovery of a novel miniplasmid in K216 was not in itself particularly surprising, for many small plasmids, often of unknown function, had been identified in wild-type isolates of different bacteria (for example, see Cozzarelli, Kelly and Kornberg, 1968; Sheehy, Allison and Curtis, 1973; Porter, Kolodner and Warner, 1973). However, the fact that this miniplasmid was mobilised from K216 was very interesting, for it had been shown that more than 1,500 base pairs of ColE1 were involved in coding its mobilisation proteins (Dougan et al., 1978; Warren et al., 1978), yet the miniplasmid of K216 was probably only about 1,500 base pairs in total length.

An investigation into the mechanism of mobilisation of this miniplasmid is presented in the following chapter. Experiments are reported here which describe the discovery and physical characterisation of this miniplasmid.

II. Initial Examination of the Plasmid Content of K216 and K235

In order to construct K-12 derivatives which carried ColK, two colicin K producing strains of *E.coli* - K216 and K235 - were considered as potential sources of the ColK plasmid. These strains were obtained from P. Reeves (see Davies and Reeves, 1975), but came originally from the collection compiled by P. Fredericq, and were both clinical isolates from apparently unrelated individuals living in the Liege region of Belgium.

Initially the plasmid content of K216 and K235 was examined by 0.5% agarose gel electrophoresis of SDS cleared lysates. As shown in figure 4.1, this revealed that whilst K216 apparently contained only the 4.3 Mdal ColK plasmid, K235 contained a larger DNA species in addition to ColK. It has been reported that K235 produces two colicins, K and X (Davies and Reeves, 1975; Hughes, Le Grice, Hughes and Meynell, 1978), yet by sucrose gradient analysis of cleared lysates (Hardy, Meynell, Dowman and Spratt, 1973; Hughes *et al.*, 1978) only the ColK plasmid has been identified. It seems likely that the novel plasmid band visible in the SDS cleared lysate of K235 corresponds to the ColX plasmid. Since K216 apparently contained only the ColK plasmid, this strain was chosen as the source of ColK in constructing ColK-carrying K-12 derivatives.

III. Discovery of a Novel Plasmid in K216

The K-12 derivative GB15/R64*drd*11 + ColK was constructed by mobilising ColK from K216, using R64*drd*11 to promote conjugation. The presence of R64*drd*11 and of the 4.3 Mdal ColK plasmid was verified by 0.5% agarose gel electrophoresis of an SDS cleared lysate. However, when a cleared lysate of this derivative was subjected to preparative neutral sucrose gradient centrifugation (in order to assay ColK relaxation

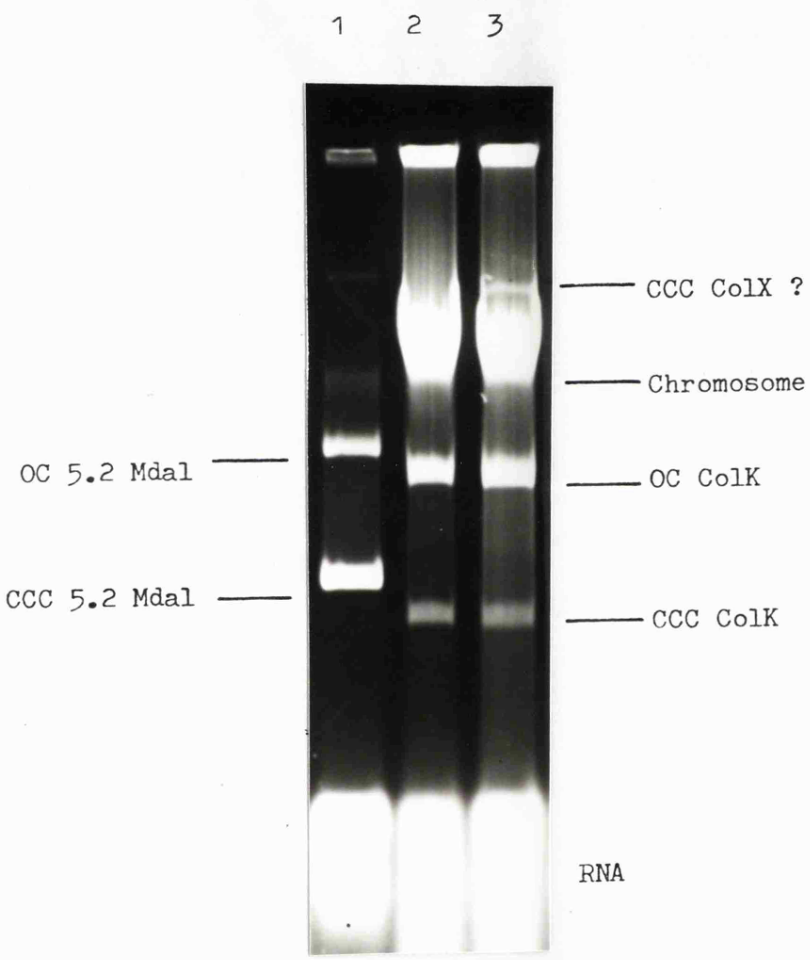
Figure 4.1: PLASMID CONTENT OF K216 AND K235

SDS plate cleared lysates of K216 and K235, and a liquid lysate of a strain carrying 5.2 Mdal and 60 Mdal plasmids, were examined by 0.5% agarose gel electrophoresis.

slot 1: 5.2 Mdal plasmid + 60 Mdal plasmid

slot 2: K216

slot 3: K235


1
2
3
CCC ColX ?
Chromosome
OC 5.2 Mdal
OC ColK
CCC 5.2 Mdal
CCC ColK
RNA

complex levels) an unusual gradient profile was obtained. In figure 4.2 the neutral sucrose gradient profiles of DNA from Triton X-100 cleared lysates of this derivative, and of a ColE1-carrying strain, are compared. Cultures of both strains were ^{3}H thymidine labelled, and then harvested in the mid log phase. Triton X-100 cleared lysates were prepared, and subjected to preparative neutral 5-20% sucrose gradient centrifugation. In the case of ColE1 a typical gradient profile, consisting of two peaks of labelled material, was obtained, but for the ColK-carrying derivative three peaks were seen. In both cases the peak material was pooled (as indicated in figure 4.2), and 30 µl aliquots of the pooled material were examined by agarose gel electrophoresis. The results of this analysis are also included in figure 4.2. As expected, the ColE1 A and B peaks contained the CCC and OC plasmid DNA respectively; similarly, for the ColK^{+} strain, C and D peaks contained CCC and OC ColK DNA. However, the ColK-carrying derivative also contained two small novel DNA species, one of which cosedimented in the sucrose gradient with OC ColK DNA, and the other which sedimented more slowly (peak E). The relative positions of the two novel DNA species in a neutral sucrose gradient and an agarose gel suggested that they might represent the CCC and OC forms of a very small plasmid.

The miniplasmid present in the K-12 derivative (GB15/R64drd11 + ColK) was presumably acquired as a result of transfer from K216. Previous examination of K216, by agarose gel electrophoresis of an SDS cleared lysate (see figure 4.1), had suggested that this strain contained only the ColK plasmid. However, it is obvious that in such an analysis the presence of any very small DNA species would be obscured by the broad band of RNA which migrates near the front of the gel. An SDS cleared lysate of K216 was therefore RNAase treated, and examined by agarose gel electrophoresis (see figure 4.2). This analysis shows that K216 does contain some small novel DNA species, and one of these is identical to

Figure 4.2: PLASMID CONTENT OF A COLK$^+$ K-12 DERIVATIVE MADE BY TRANSFER OF THE COLK PLASMID FROM K216

(a) The neutral sucrose gradient profiles of Triton X-100 cleared lysates of (i) a ColE1-carrying strain, and (ii) the ColK-carrying derivative, were compared.

(b) An RNAase treated SDS cleared lysate of K216, and material pooled from each of the peaks A, B, C, D and E (part a), were examined by 0.8% agarose gel electrophoresis.

slot 1: K216, RNAase treated

slot 2: 30 µl pool A

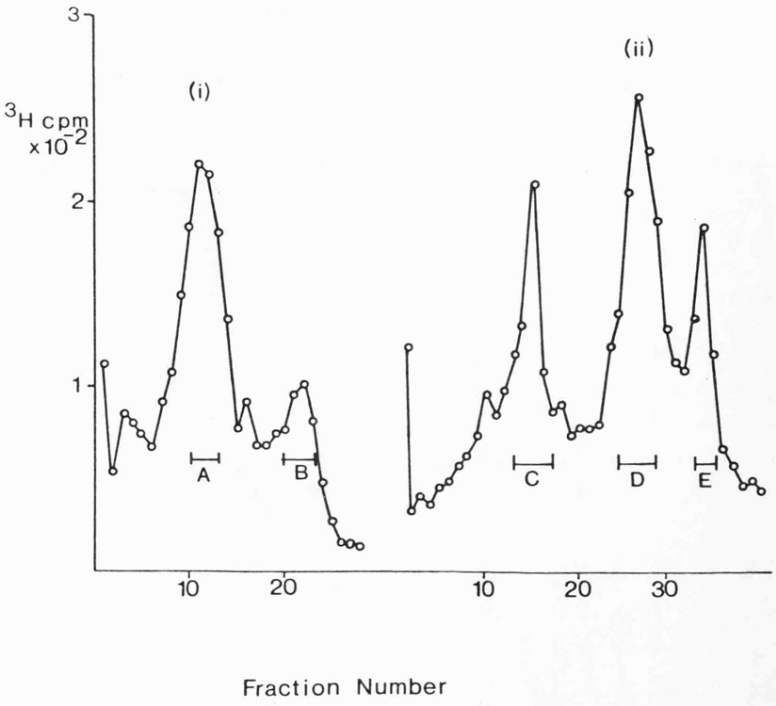
slot 3: 30 µl pool B

slot 4: 30 µl pool C

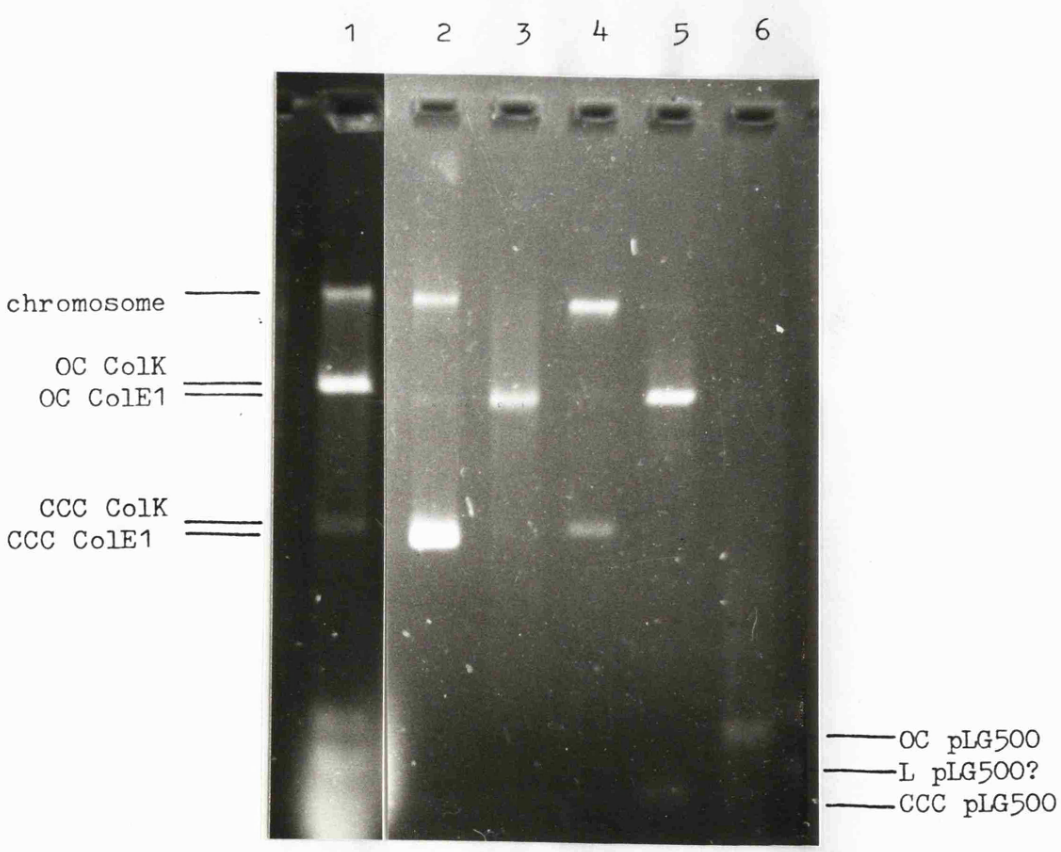
slot 5: 30 µl pool D

slot 6: 30 µl pool E

(a)


3
2
1
^{3}H cpm
x10^{-2}
(i)
(ii)
A
B
C
D
E
10
20
10
20
30
Fraction Number

(b)


1
2
3
4
5
6
chromosome
OC ColK
OC ColE1
CCC ColK
CCC ColE1
OC pLG500
L pLG500?
CCC pLG500

the slower migrating form present in the K-12 derivative.

These results indicate that K216 contains one or more naturally occurring miniplasmids. It seems likely that the two novel DNA species present in K216 and in the K-12 derivative are different conformations of the same miniplasmid. In accordance with the proposals of Novick, Clowes, Cohen, Curtiss, Datta and Falkow (1976), I have called this miniplasmid pLG500.

IV. Identification of the Different Molecular Forms of pLG500

Supercoiled pLG500 plasmid DNA was isolated from GB15/R64drd11 + ColK + pLG500 by CsCl-EtBr gradient centrifugation of a Triton X-100 cleared lysate, and then separated from supercoiled ColK DNA by preparative neutral sucrose gradient centrifugation. The CCC, OC, and linear (L) forms of pLG500 were identified by performing controlled DNAase I nicking, and HaeIII restriction endonuclease digestion, of the purified plasmid DNA. DNAase I introduces random single-stranded nicks into DNA, so that CCC plasmid DNA is converted to the OC, and eventually to the L, form. HaeIII restriction of pLG500 yields only a single digestion product, and therefore converts CCC pLG500 DNA to the L form. Digestion products were examined by agarose gel electrophoresis as shown in figure 4.3. It was found that DNAase I converted the putative CCC form to a slower migrating form, and this was eventually replaced by a form with intermediate mobility. Therefore, in order of increasing mobility, the three forms of pLG500 were OC, L, and CCC. HaeIII digestion, yielding linearised pLG500 DNA, confirmed that the form with intermediate mobility was the linear form.

Figure 4.3: IDENTIFICATION OF THE CCC, OC, AND L FORMS OF PLG500

Pancreatic DNAase I, and HaeIII restriction endonuclease, digestion products of pLG500 were subjected to 1.8% agarose gel electrophoresis.

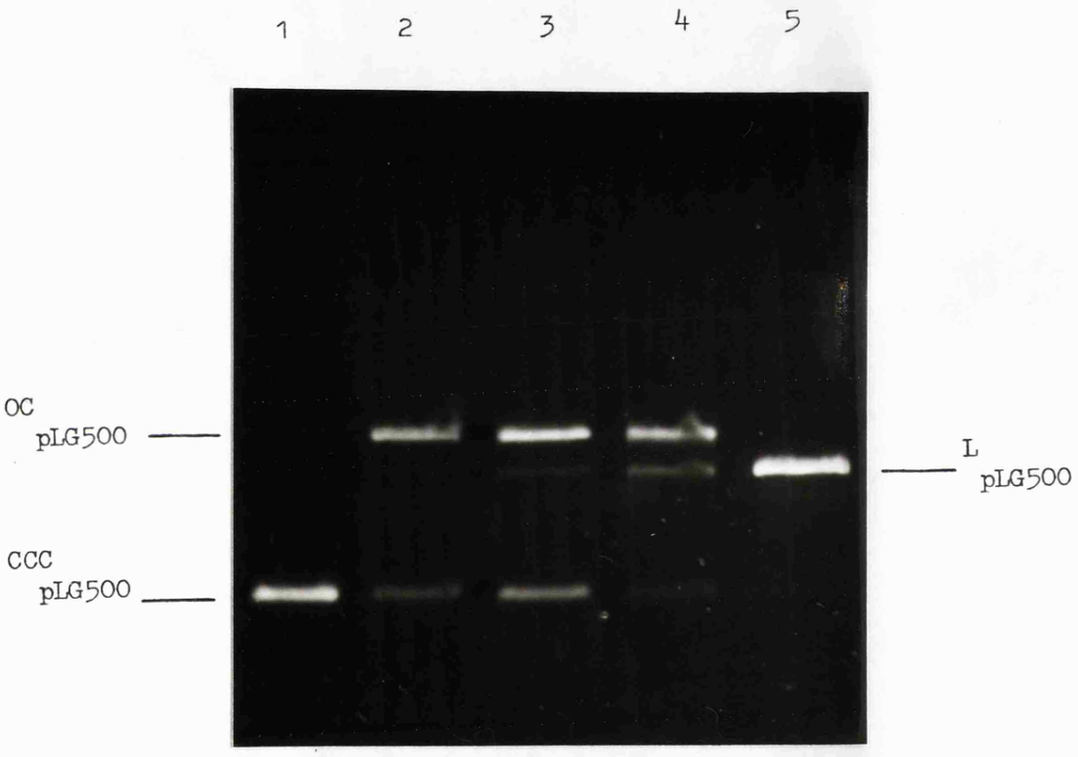
slot 1: undigested pLG500

slot 2: pLG500 x DNAase I, $\frac{1}{2}$ minute at 37°C

slot 3: " 2 minutes at 37°C

slot 4: " 10 minutes at 37°C

slot 5: pLG500 x HaeIII


1
2
3
4
5
OC
pLG500
CCC
pLG500
L
pLG500

V. Size of pLG500

In order to estimate the size of pLG500, the mobility of its linear form was compared to the mobilities of linear restriction fragments of known size, in an agarose gel. Since the entire nucleotide sequence of pBR322 has been determined (Sutcliffe, 1978), restriction endonuclease digestion products of this plasmid were used as molecular weight markers. The HaeIII digestion product of pLG500, and the HaeII and TaqYI digestion products of pBR322, were subjected to 1.8% agarose gel electrophoresis (see figure 4.4). pBR322 A and B TaqYI restriction fragments are 1,444 and 1,307 bases long; the A and B HaeII fragments are 1,876 and 622 bases long. Therefore a good estimate of the size of pLG500 is 1,500 base pairs.

VI. Autonomous Replication of pLG500

Since pLG500 was one of the smallest naturally occurring plasmids ever found, experiments were performed to determine whether it was an autonomous replicon. In a series of mobilisation experiments (described in a subsequent chapter), it was found that pLG500 could be mobilised from a donor strain carrying ColE1 and the incompatibility P group plasmid, RP4. In a mating with the plasmidless recipient GB15, some R plasmid transconjugants received ColE1 and pLG500, whereas some only received ColE1 or pLG500. Since cells which carry RP4 are sensitive to infection by P group plasmid pilus-specific phages, it was possible to select for spontaneous loss of the RP4 plasmid from a population. The strain GB15/RP4 + pLG500 was subjected to phage PRR1 infection by spreading 0.1 ml of an overnight culture onto a nutrient agar plate and streaking a PRR1 phage preparation across the plate. Any survivors of PRR1 exposure were tested for sensitivity to the antibiotics tetracycline and kanamycin, for which RP4 codes

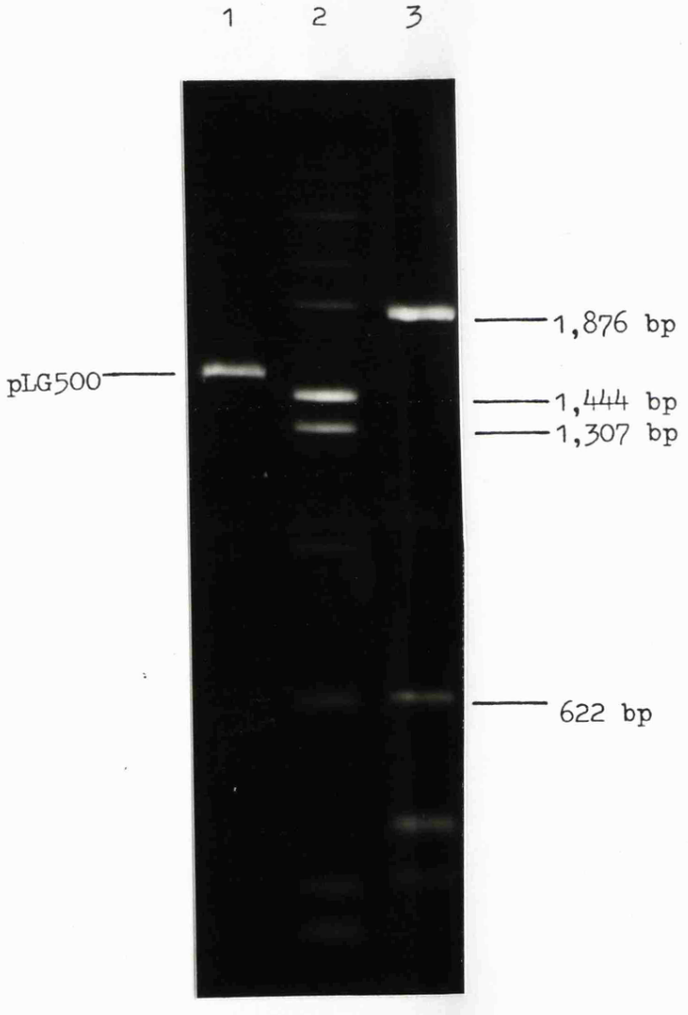
Figure 4.4: DETERMINATION OF THE SIZE OF PLG500

The linear form of pLG500, and pBR322 restriction fragments of known size, were subjected to 1.8% agarose gel electrophoresis.

slot 1: pLG500 x HaeIII

slot 2: pBR322 x TaqY1

slot 3: pBR322 x HaeII


1
2
3
pLG500
1,876 bp
1,444 bp
1,307 bp
622 bp

non-transposable drug resistance. In this way several isolates which had spontaneously lost the RP4 plasmid were obtained. RNAase treated SDS cleared lysates of these isolates, and of the parental strain GB15/RP4 + pLG500, were examined by agarose gel electrophoresis (see figure 4.5). An RNAase treated SDS cleared lysate of the wild-type strain, K216, from which pLG500 came originally, was also included. Figure 4.5 demonstrates that pLG500 is maintained in cells which carry no other plasmids, and it must therefore be capable of autonomous replication. It is noteworthy that pLG500 alone, or in the presence of RP4, exists only in the CCC form, whereas in K216 most of the pLG500 DNA is in the OC and L forms. This aspect will be discussed in detail in the following chapter.

Finally the strain GB15/pLG500 was tested for colicin production, immunity to colicins E1, E2, and K, and for resistance to a range of common antibiotics. It was found that pLG500 does not mediate colicin production or immunity, and does not encode any common drug resistances.

VII. Summary

pLG500 is a naturally occurring miniplasmid present in the colicin K producing clinical isolate of *E.coli*, K216. This miniplasmid is an autonomous replicon with a molecular weight of about one million. Whilst pLG500 is a cryptic plasmid in that it has no discernible phenotype, it is however capable of being transferred from K216 during R64*drd*11 mediated conjugation.

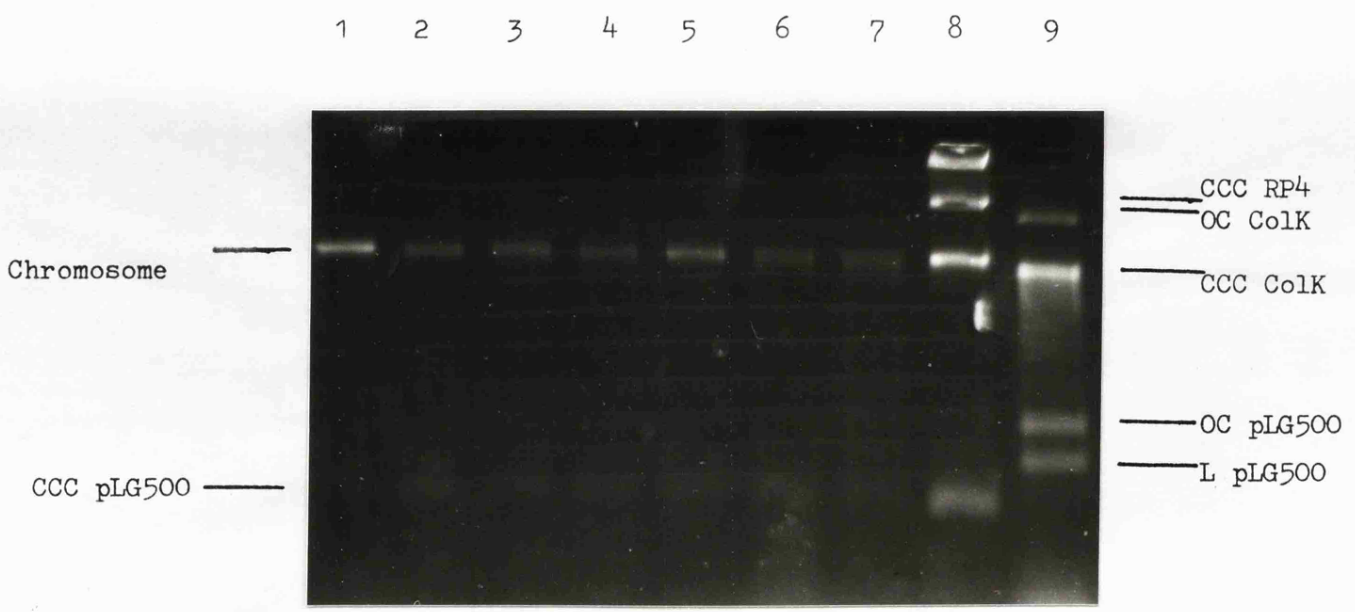
Figure 4.5: AUTONOMOUS REPLICATION OF PLG500

RNAase treated SDS cleared lysates of GB15/RP4 + pLG500, derivatives of this strain which had lost the RP4 plasmid, and K216, were analysed by 1.2% agarose gel electrophoresis.

slots 1-7: Tc^S, Kan^S, derivatives of GB15/RP4 + pLG500

slot 8: GB15/RP4 + pLG500

slot 9: K216


1
2
3
4
5
6
7
8
9
Chromosome
CCC pLG500
CCC RP4
OC ColK
CCC ColK
OC pLG500
L pLG500

CHAPTER 5

REQUIREMENTS FOR MOBILISATION AND RELAXATION OF pLG500

I. Introduction

Since pLG500 was transferred from K216, during R64drd11 mediated conjugation, despite the fact it was very small, this suggested either that it directed its own mobilisation by some novel mechanism which was quite different to that of ColE1, or that it depended upon the provision of ColK-coded gene products for its transfer. Therefore in this chapter an analysis of the requirements for pLG500 mobilisation is presented.

II. Efficiency of Mobilisation of pLG500 in the Presence and Absence of ColK

Since the efficiency of R64drd11 promoted mobilisation of ColK was known to be high (for example, see table 3.2), and, in the case of GB15, receipt of ColK from K216 was accompanied by receipt of pLG500, this suggested that pLG500 was also efficiently mobilised from K216. A comparison of ColK and pLG500 mobilisation frequencies was made by mating the donor strain GB15/R64drd11 + ColK + pLG500 with the plasmidless recipient JB2. It was found that following a 30 minute mating, 90% of the R plasmid transconjugants had acquired the ability to produce colicin, and of 50 R plasmid transconjugants directly examined for the presence of pLG500, all of these contained the miniplasmid. These results indicated that ColK and pLG500 were both very efficiently mobilised from the donor strain GB15/R64drd11 + ColK + pLG500, and that the miniplasmid was even more efficiently mobilised than ColK.

To determine whether pLG500 coded all the gene products necessary for its mobilisation, R plasmid transconjugants which had received either ColK and pLG500, or only pLG500, were purified, and then used as donors in matings with the plasmidless, colicin K resistant, recipient JB1. In the case of the ColK$^+$ donor (JB1/R64drd11 + ColK + pLG500), it was found that both ColK and pLG500 were efficiently mobilised; but when ColK was absent from the donor (JB1/R64drd11 + pLG500), of 50 R plasmid transconjugants examined, none were found to have received pLG500. These results demonstrated that the mobilisation of pLG500 was absolutely dependent upon the presence of ColK in the donor. Since (in the former cross) R plasmid transconjugants which had received only pLG500 could be found, and since those which had received ColK and pLG500 contained both of the intact parental plasmids, this showed that mobilisation of pLG500 depended upon the provision of ColK-coded gene products, rather than physical attachment of pLG500 DNA to ColK in the donor.

III. Ability of ColK, ColE1, and ColE2 to Complement the Mobilisation Deficiency of pLG500

The fact that pLG500 was mobilised when ColK-coded gene products were supplied in *trans* suggested that this miniplasmid was analogous in its mobilisation properties to those Mob$^-$ derivatives of ColE1 which were defective in mobilisation protein production. Therefore, although pLG500 is a naturally occurring plasmid, it can be said to have a 'mobilisation deficiency'. Since ColK complemented the mobilisation deficiency of pLG500, the ability of the closely related plasmid ColE1, and of an unrelated mobilisation proficient plasmid ColE2, to complement this deficiency was examined. Isogenic donor strains carrying R64drd11, pLG500, and ColK, ColE1, or ColE2, were constructed. A *recA* donor strain (GB15) was used to exclude any recombination between pLG500 and

the various Col plasmids, due to possible sequence homology. Matings with the plasmidless recA recipient AB2463 were performed as described in Chapter 2. The mobilisation frequencies of the Col plasmids were determined by scoring 200-400 R plasmid transconjugants for immunity to the appropriate colicin. The efficiency of mobilisation of pLG500 was determined by direct examination of the plasmid content of 50 R plasmid transconjugants from each cross. The results of these matings are summarised in table 5.1. These experiments confirm that pLG500 does not code all the gene products necessary for its mobilisation, however, both of the closely related ColK and ColE1 plasmids can complement the mobilisation deficiency of this miniplasmid. In contrast ColE2, which is itself efficiently mobilised during R64drd11 promoted conjugation but is unrelated to ColK and ColE1 plasmids, cannot complement the mobilisation deficiency of pLG500.

IV. Relaxation Nicking of pLG500

(a) Ability of ColK, ColE1, and ColE2 to Complement the Relaxation Deficiency of pLG500

In the mating experiments summarised in table 5.1, R plasmid transconjugants were screened for the presence of pLG500 by 1.2% agarose gel electrophoresis of RNAase treated SDS cleared lysates. During this screening procedure it became apparent that the conformation of pLG500 plasmid molecules in the recipients varied depending on whether ColK or ColE1 plasmids were also present. Similarly, in the previous chapter I pointed out that the conformation of pLG500 in an SDS cleared lysate of K216 was quite different to the conformation in isolates which carried pLG500 and RP4, or pLG500 alone. Since, in addition to lysing cells, SDS treatment induces relaxation nicking of any plasmid present in the form of a relaxation complex, then in SDS cleared lysates any

Table 5.1

COMPLEMENTATION OF THE MOBILISATION DEFICIENCY OF PLG500

The recA donor strains were mated with the recA recipient AB2463 for 30 minutes, as described in chapter 2. Recipients of R64drd11 were then selected: 200-400 of these were tested for immunity to the appropriate colicin, and 50 were examined for the presence of pLG500 by 1.2% agarose gel electrophoresis of RNAase treated SDS cleared lysates.

Donor GB15/R64drd11	Efficiency of Mobilisation	
	Col Plasmid	pLG500
+ pLG500	—	0
+ pLG500 + ColK	90	100
+ pLG500 + ColE1	95	100
+ pLG500 + ColE2	98	0

supercoiled pLG500 plasmid DNA present as a relaxation complex will be converted to the OC form. 1.2% agarose gel electrophoresis of RNAase treated SDS cleared lysates of each of the donor strains (table 5.1) is presented in figure 5.1. The results demonstrate that pLG500 in the presence of R64drd11 alone cannot be induced to relax by SDS treatment. However, if ColK or ColE1 is also present SDS treatment converts most of the pLG500 DNA to the OC form. In contrast ColE2, which is itself a relaxable plasmid (for example, see Blair et al., 1971; Lovett et al., 1974a), does not render pLG500 relaxable by SDS treatment. In conclusion, pLG500 must possess a site (or sites) at or near which ColK and ColE1 relaxation proteins can bind and facilitate relaxation nicking.

(b) Relaxability of pLG500 extracted from Cells grown using Glucose or Glycerol as the Carbon Source

Cultures of strains carrying pLG500 alone, or plus R64drd11, ColK, and ColE1, were grown overnight in minimal medium containing 0.5% casamino acids, with 0.2% glucose or 0.2% glycerol as the carbon source. SDS cleared lysates of cells were prepared, and then RNAase treated, and subjected to 1.2% agarose gel electrophoresis, as shown in figure 5.2. This experiment demonstrates that pLG500 is unrelaxable, regardless of the carbon source used, when it exists alone, or in the presence of R64drd11 only. However, if ColK or ColE1 is also present pLG500 is rendered relaxable, and in both cases substantially more of the pLG500 plasmid DNA is relaxable in extracts from glycerol rather than glucose grown cells.

Figure 5.1: CONFIGURATIONS OF PLG500 PLASMID DNA IN THE PRESENCE OF COLK, COLE1, OR COLE2, FOLLOWING SDS-INDUCED RELAXATION NICKING

RNAase treated SDS cleared lysates of the donor strains (see table 5.1) were analysed by 1.2% agarose gel electrophoresis.

slot 1: GB15/R64drd11 + pLG500

slot 2: GB15/R64drd11 + ColK + pLG500

slot 3: GB15/R64drd11 + ColE1 + pLG500

slot 4: GB15/R64drd11 + ColE2 + pLG500

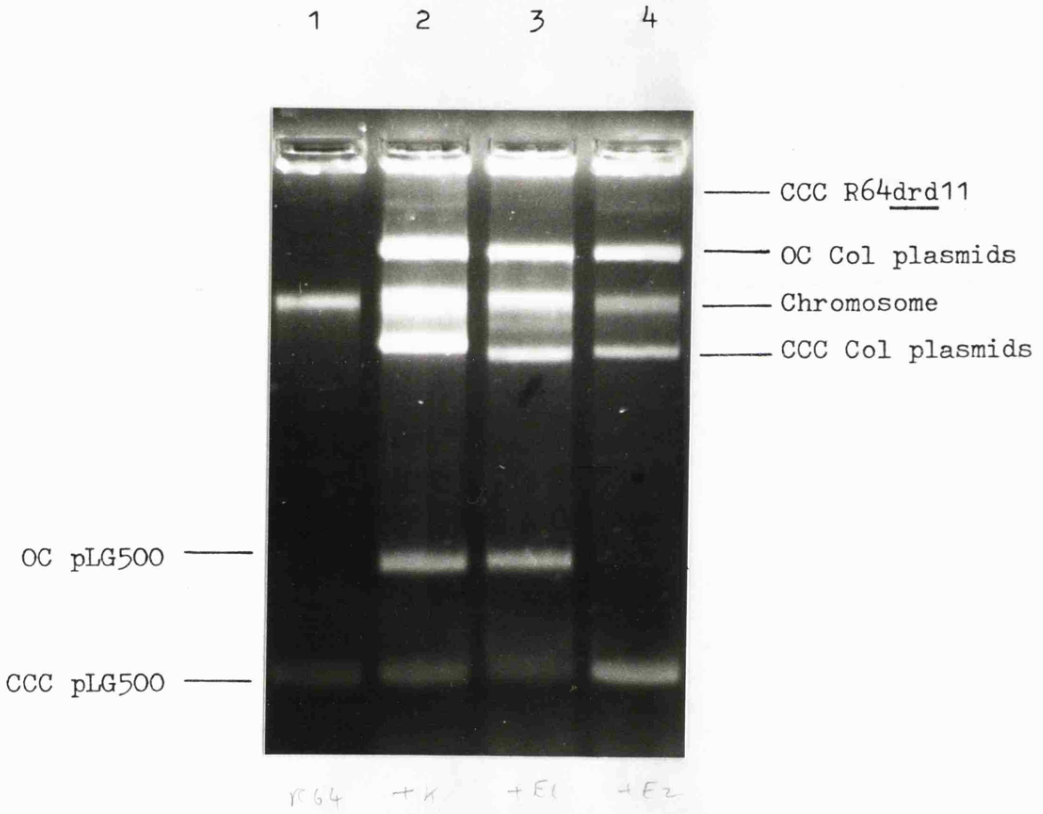

1
2
3
4
CCC R64drd11
OC Col plasmids
Chromosome
CCC Col plasmids
OC pLG500
CCC pLG500
R64
+K
+E1
+E2

Figure 5.2: SDS-INDUCED RELAXABILITY OF PLG500 FROM CELLS GROWN WITH EITHER GLUCOSE OR GLYCEROL AS THE CARBON SOURCE

RNAase treated SDS cleared lysates were made from 5 ml L broth overnight cultures, and examined by 1.2% agarose gel electrophoresis.

slot 1: GB15/pLG500, glycerol grown

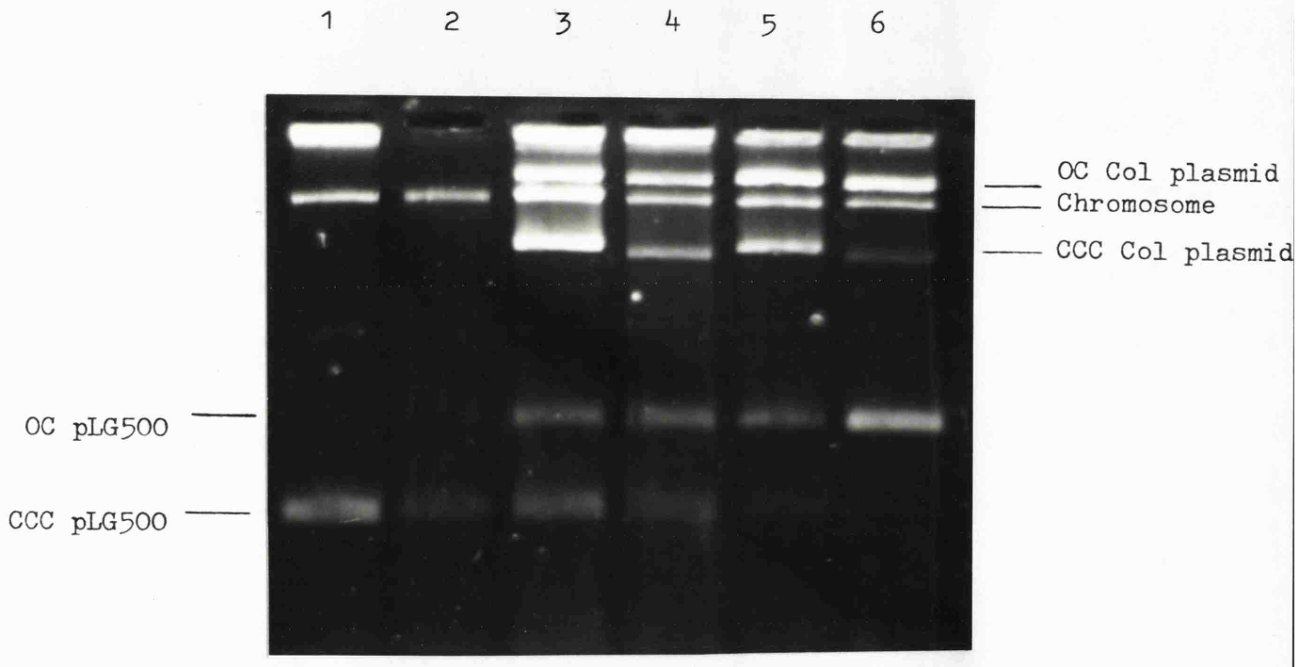
slot 2: GB15/R64drd11 + pLG500, glycerol grown

slot 3: GB15/R64drd11 + ColK + pLG500, glucose grown

slot 4: GB15/R64drd11 + ColE1 + pLG500, glucose grown

slot 5: GB15/R64drd11 + ColK + pLG500, glycerol grown

slot 6: GB15/R64drd11 + ColE1 + pLG500, glycerol grown


1
2
3
4
5
6
OC Col plasmid
Chromosome
CCC Col plasmid
OC pLG500
CCC pLG500

V. Discussion

pLG500 is a naturally occurring miniplasmid which, in the presence of the conjugative plasmid R64drd11 alone, cannot be mobilised and is non-relaxable. However, if either of the two closely related plasmids, ColK and ColE1, are also present, they can supply gene products which efficiently complement the mobilisation and relaxation deficiency of pLG500. In contrast, the unrelated plasmid ColE2 is itself mobilisable and relaxable, but cannot complement the mobilisation or relaxation deficiency of pLG500. Since ColK and ColE1 complement both the mobilisation and relaxation deficiency of pLG500, whereas ColE2 complements neither, this correlation supports the view that relaxation complexes are involved in plasmid mobilisation. These results strongly suggest that relaxation nicking of pLG500, which can only occur in the presence of ColK or ColE1-coded gene products, is a prerequisite for the mobilisation of this miniplasmid.

Complementation of the pLG500 mobilisation deficiency by ColK or ColE1 results in a higher frequency of mobilisation of the miniplasmid than either of the Col plasmids. Presumably the efficiency of mobilisation of a plasmid will depend both on its total copy number as well as the proportion of molecules which can be relaxed. Although I have not accurately measured the copy number of pLG500, various estimates suggest that it greatly exceeds that of ColK or ColE1, and this difference may account for its greater efficiency of mobilisation. However in the gels shown in figure 5.1 and 5.2, complementation of the pLG500 relaxation deficiency apparently results in a greater proportion of pLG500 plasmid DNA being relaxed than either of the Col plasmids. Whether this result is an artefact (perhaps related to the different sizes of these plasmids), or whether pLG500 has a greater affinity than ColK or ColE1 DNA for their relaxation proteins, is not clear.

Although I found previously (chapter 3) that ColK did not exist in the form of a relaxation complex, the fact that pLG500 is rendered relaxable in the presence of this plasmid demonstrates that ColK does code relaxation protein equivalents. Possibly the trans activity of the ColK 'relaxation' proteins can be demonstrated in this way (that is, as shown in figure 5.1) because a relaxation complex purification step, during which proteins of the complex might dissociate from the supercoiled ColK plasmid DNA, is not necessary. The results presented in figure 5.2 demonstrate that the trans activity of ColK and ColE1 relaxation proteins on pLG500 is very similar. In both cases more relaxation proteins are produced when glycerol rather than glucose is used as the carbon source, and slightly more of the pLG500 DNA is rendered relaxable in the presence of ColE1 rather than ColK.

I showed in the last chapter that there was a glucose-glycerol effect on the level of ColK mobilisation, and on the efficiency of mobilisation of pML21 when ColK complemented its mobility defect. The results presented here show, albeit indirectly, that there is also a glucose-glycerol effect on the level of trans-acting ColK relaxation proteins. Therefore these experiments provide further support for a correlation between the efficiency of plasmid mobilisation and relaxation. In the SDS cleared lysates of figure 5.2, pLG500 clearly displayed increased relaxability in the presence of ColK; previously however no stimulation of pML21 relaxability by ColK could be demonstrated (see chapter 3, section IV). This difference may be attributed in part to the fact that pLG500 exists only in the supercoiled form even after SDS treatment, whereas pML21 exhibits similar levels of open circular DNA to ColK. Therefore for pLG500 any increase in the proportion of open circular material is very obvious, whereas for pML21 this may be more difficult to detect.

Since the two closely related plasmids ColK and ColE1 complement the mobilisation and relaxation deficiency of pLG500, whereas the unrelated plasmid ColE2 does not, this suggests that pLG500 is closely related to ColK and ColE1 in terms of sequence similarity. Several other naturally occurring miniplasmids have been shown to share sequence homology with ColE1. The 1.45 Mdal (P15A) miniplasmid which was isolated from E.coli strain 15 (Cozzarelli et al., 1968) has since been shown in hybridisation studies to be largely homologous with ColE1 (Goebel and Schrempf, 1972). In the same study two plasmids, of 1.8 Mdal and 2.3 Mdal, from different isolates of E.coli found in ox intestine, were shown to be substantially homologous with the P15A miniplasmid. In addition to these, a 3.5 Mdal plasmid has been found in Shigella dysenteriae strain Y6R, which appears to be a defective ColE1 plasmid (Porter et al., 1973). It seems likely therefore that there are many, as yet unidentified, miniplasmids which are related to ColE1. In turn, it may be that most small plasmids fall into only a few groups in terms of sequence similarity.

The P15A miniplasmid of E.coli 15 is the most extensively studied of these naturally occurring miniplasmids. Recently Chang and Cohen (1978) have shown that this plasmid is compatible with ColE1, and is rendered mobilisable in its presence. P15A and pLG500 are therefore similar in that they both possess a bom site which can be recognised by ColE1-coded proteins, but do not themselves code a full complement of gene products necessary for their mobilisation. Previously Messing, Staudenbauer and Hoffschneider (1972; 1973) showed that P15A could be isolated as a supercoiled DNA-protein complex which possessed an endonuclease activity. However this complex could only be isolated in low salt conditions, and the endonuclease activity, which required Mg^{++}, was not strand-specific. Whatever the nature of this complex, it certainly

differs from a relaxation complex. Moreover, I would predict, by analogy with pLG500, that complementation of the P15A mobilisation deficiency by ColE1 would also render the P15A miniplasmid relaxable.

Assuming that pLG500, P15A, and the other naturally occurring miniplasmids mentioned here are all ancestrally related to ColK and ColE1, then if they retain the ancestral replication origin, it seems likely that most of them will also retain the relaxation site (since for ColE1 it is only 270 bases away from the replication origin). Therefore an important consequence of these sites being close together is that most small derivatives retain the ability to be mobilised provided a closely related, mobilisation proficient, plasmid is also present. In the case of these naturally occurring miniplasmids which have no apparent selective advantage, the ability to be mobilised may therefore constitute a major survival factor.

CHAPTER 6

SITE-SPECIFIC, RECA INDEPENDENT, RECOMBINATION BETWEEN COLK AND PLG500, DETECTED FOLLOWING TRANSFER

I. Introduction

E.coli K-12 derivatives which carried ColK were originally constructed by mobilising ColK from the wild type strain K216, using R64*drd*11 to promote conjugation. K216 was chosen as the source of ColK because it appeared to contain only the ColK plasmid. Transfer of R64*drd*11 from a C600 donor strain into K216 had occurred only at a very low frequency, suggesting that K216 encoded a different host restriction modification system to *E.coli* K-12. Therefore matings from K216/R64*drd*11 were performed using a restrictionless intermediate donor (5K), and a final K-12 recipient (GB15). Since the overall mobilisation frequency of ColK was not known the final recipients were selected on the basis of immunity to colicin K, by plating the mating mixture onto selective plates spread with 0.1 ml of a crude colicin K preparation. However, when 8 colicin K immune recipients were examined by agarose gel electrophoresis of SDS cleared lysates, only 4 of them were found to contain the original (4.3 Mdal) ColK plasmid, whilst the other 4 harboured a novel plasmid which was about 1 Mdal larger than ColK.

The discovery of novel plasmids which apparently conferred colicin K immunity prompted a detailed examination of the plasmid content of the strains involved, and led to the discovery of the cryptic miniplasmid pLG500 in the wild type strain K216. Since further studies with pLG500 showed that this miniplasmid was efficiently mobilised in the presence of ColK, and that it was 1 Mdal in weight, this suggested the possibility that the novel plasmids were formed by additive recombination between

ColK and pLG500. Moreover, since there was no indication that recombination occurred between ColK and pLG500 in the Rec^+ strain K216 (for example, see figure 4.1), it was possible that the formation of these novel plasmids involved some process other than reciprocal recombination due to sequence homology. Furthermore, the new plasmids had been detected following conjugation, and ColK and pLG500 plasmids were known to be intimately related with regard to their mobilisation and relaxation properties, thus raising the possibility that the formation of these plasmids might be specifically connected with the mobilisation process.

In this chapter I shall describe experiments which confirm that these novel colicin K immune plasmids are formed by recombination between ColK and pLG500, and will describe the salient features of the recombination event which generates them.

II. Restriction analysis of ColK, pLG500, and a novel colicin K immune plasmid

Isogenic recipients of ColK and of a novel colicin K immune plasmid, which I have designated pLG520, were used to prepare supercoiled plasmid DNA. Since both recipients were found to have also received pLG500, ^3H thymidine labelled CCC DNA was prepared from GB15/R64*drd*11 + ColK + pLG500 and from GB15/R64*drd*11 + pLG520 + pLG500 by CsCl-EtBr gradient centrifugation of Triton X-100 cleared lysates, and in each case the two different CCC plasmid DNA species were separated by preparative neutral sucrose gradient centrifugation.

The physical relationship between ColK, pLG500, and pLG520 was determined by comparing their *Hae*II restriction endonuclease digestion products, analysed by agarose gel electrophoresis. As shown in figure 6.1 pLG520 is the product of cointegration of ColK and pLG500. Since pLG520 contains all the *Hae*II restriction fragments present in ColK and pLG500

Figure 6.1: HaeII RESTRICTION ANALYSIS OF COLK, PLG500, AND PLG520

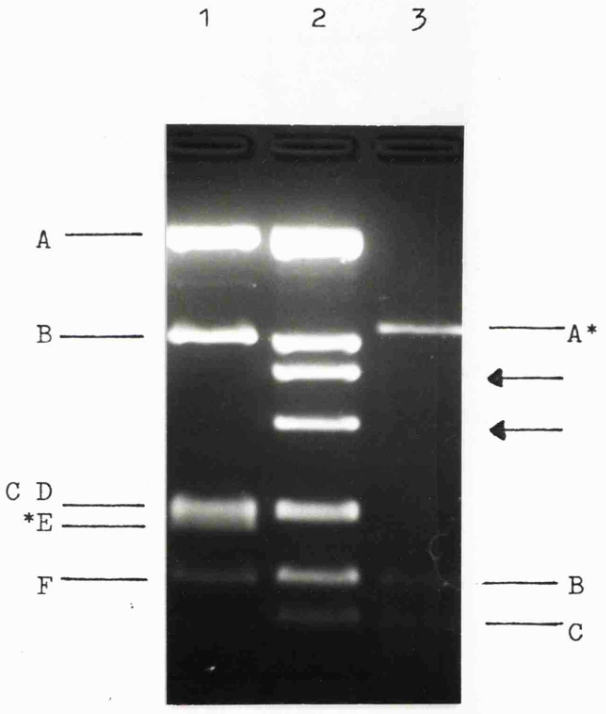
Supercoiled plasmid DNA was prepared from GB15/R64drd11 + ColK + pLG500 and GB15/R64drd11 + pLG520 + pLG500 as described in the text. The purified plasmid DNA preparations were then digested with HaeII restriction endonuclease, and analysed by 2.0% agarose gel electrophoresis.

slot 1: ColK x HaeII

slot 2: pLG520 x HaeII

slot 3: pLG500 x HaeII

The fragments of ColK and pLG500 which are absent in pLG520 are marked with asterisks. The two novel fragments in pLG520 are indicated by arrows.


1
2
3
A
B
C D
*E
F
A*
B
C

except the ColK-E and pLG500-A fragments, this demonstrates that recombination between ColK and pLG500 must have occurred at sites within these fragments. Since the ColK-E and pLG500-A fragments are replaced by two novel fragments of the same total size, this indicates that recombination occurs without the loss or duplication of any ColK or pLG500 plasmid DNA.

III. Recombination between ColK and pLG500 is only detected following transfer, and it occurs recA independently

Since ColK-pLG500 plasmids were found following the transfer of ColK and pLG500 from K216, yet there was no detectable recombination between them in K216, it was possible that recombination occurred specifically during the comobilisation of ColK and pLG500. Alternatively, it might be that recombination between ColK and pLG500 occurred in vegetatively growing cells, but that such recombinants were rare and were only detected if they were subsequently transferred to a plasmidless recipient.

To determine whether recombination between ColK and pLG500 was only detectable following transfer, and whether it might simply be due to generalised recombination between these two plasmids, which were already known to be closely related genetically, the following mating experiments were performed. The recA donor strain GB15/R64drd11 + ColK + pLG500 was mated with the recA recipient AB2463, and at the same time the Rec^+ donor 5K/R64drd11 + ColK + pLG500 was mated with the Rec^+ recipient JB1. In each case 50 unmated donor colonies, and 50 R plasmid transconjugants, were examined by 0.5% agarose gel electrophoresis of SDS cleared lysates. The plasmid content of some recipients of the cross GB15/R64drd11 + ColK + pLG500 x AB2463 is shown in figure 6.2. The results of these two mobilisation experiments are summarised in table 6.1. In both cases it was found that R plasmid transconjugants

Figure 6.2: APPEARANCE OF R PLASMID TRANSCONJUGANTS OF COLK AND PLG500 COMOBILISATION

The plasmid content of R plasmid transconjugants of the cross GB15/R64drd11 + ColK + pLG500 x AB2463 was examined by 0.5% agarose gel electrophoresis of SDS cleared lysates. Part of a screening gel, showing two recipients that have acquired ColK-pLG500 plasmids (slots 1 and 5) instead of ColK (all other slots) is shown.

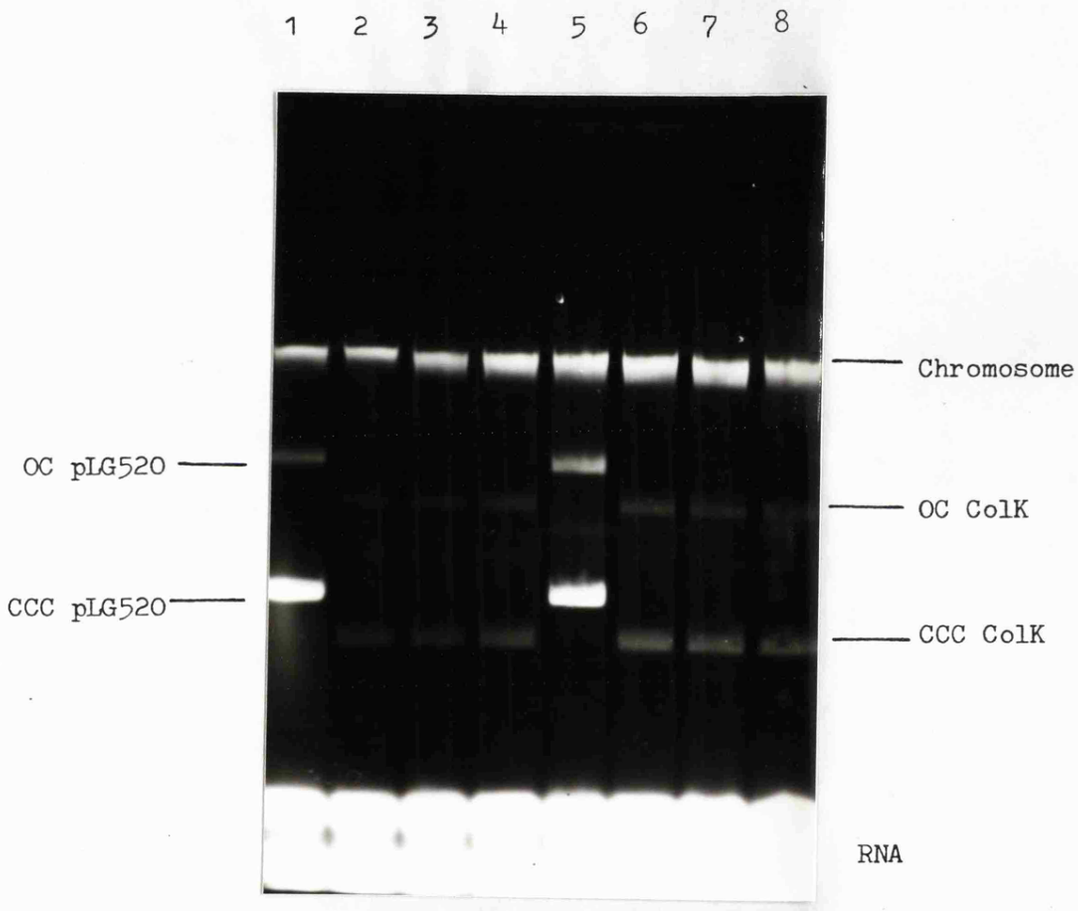

1 2 3 4 5 6 7 8
OC pLG520
CCC pLG520
Chromosome
OC ColK
CCC ColK
RNA

Table 6.1

FREQUENCY OF COMPOSITE PLASMID FORMATION DETECTED FOLLOWING COMOBILISATION OF COLK AND PLG500 BETWEEN REC^+, OR RECA, DONORS AND RECIPIENTS

The Rec^+, or *recA*, donor and recipient were mated for 30 minutes, as described in chapter 2. R plasmid transconjugants were then selected, and 50 from each cross were examined for the presence of ColK-pLG500 plasmids by 0.5% agarose gel electrophoresis of SDS cleared lysates.

Donor (R64<u>drd</u>11 + ColK + pLG500)	Recipient	R Plasmid Transconjugants ColK$^+$	ColK-pLG500$^+$	Col$^-$
Rec$^+$ - 5K	Rec$^+$ - JB1	47	1	2
<u>recA</u> - GB15	<u>recA</u> - AB2463	43	2	5

occasionally received ColK-pLG500 plasmids instead of ColK, whereas the unmated donor colonies were all identical in plasmid content, and apparently only ever contained ColK and pLG500.

The fact that ColK-pLG500 plasmids were detected following the comobilisation of ColK and pLG500 from a recA donor to a recA recipient demonstrates that their cointegration is the result of an illegitimate recombination event. Of the 100 recipients examined in these experiments only 3 were found to contain ColK-pLG500 plasmids, and therefore it is difficult to make a useful comparison of the frequency with which cointegration occurs following comobilisation from a recA or Rec^+ donor. However, since significantly greater numbers of composite plasmids were not detected amongst recipients following mobilisation from the Rec^+ donor this strongly suggests that recombination due to sequence homology either does not occur between ColK and pLG500, or, if it does, such plasmids are very rarely, if ever, transferred to recipients.

IV. Recombination between ColK and pLG500 is site-specific

In subsequent mobilisation experiments it became apparent that different ColK-pLG500 plasmids were not only all of an identical size but were also similar to one another, but strikingly different to ColK, in appearance. Both of the ColK-pLG500 plasmids shown in figure 6.2 are less relaxable, and apparently possess a higher copy number, than ColK. Their similarity raises the question of whether the recombination event which generated them might be site-specific. Therefore six ColK-pLG500 plasmid recipients were independently isolated (in four different mobilisation experiments) and supercoiled plasmid DNA was prepared from each isolate so that the ColK-pLG500 plasmid restriction patterns could be compared. In each case the HaeII restriction pattern was found to be identical to that of the original pLG520 plasmid shown in figure 6.1. The identity of these restriction patterns indicated that

the *recA* independent recombination event which resulted in the formation of pLG520 plasmids involved unique sites in the ColK and pLG500 genomes. These results also showed (unless sites for recombination were situated mid way along both the ColK-E and pLG500-A *HaeII* restriction fragments) that ColK and pLG500 plasmids always recombined in the same relative orientations.

V. Reversibility of recombination between ColK and pLG500

Once formed pLG520 plasmids were apparently stable, for no evidence of dissociation to yield the component replicons, either in SDS cleared lysates (for example, see figure 6.2) or in plasmid DNA prepared by CsCl-EtBr gradient centrifugation, could be found. However when colicin K immune R plasmid transconjugants of pLG520 mobilisation were examined, some of them were found to have received a plasmid identical in size (and indistinguishable in appearance) to the original ColK plasmid, instead of the parental plasmid pLG520. In one case plasmid DNA was prepared, and *HaeII* restriction analysis was performed, confirming that this plasmid was identical to the original ColK plasmid.

In table 6.2 the results of mating experiments comparing the mobilisation of pLG520 or of ColK and pLG500, during R64*drd11* promoted conjugation, are shown. The efficiency of mobilisation of the Col plasmids was determined as the percentage of R plasmid transconjugants which were colicin K immune. Evidence of recombination between ColK and pLG500 was sought by examining 50 colicin K immune recipients of each cross for the presence of ColK or pLG520. Since some recipients of pLG520 mobilisation received ColK instead of pLG520 this demonstrates that the recombination event which generated the composite plasmid is reversible. In addition these results show that pLG520 is less efficiently mobilised than ColK.

Table 6.2

FREQUENCY OF RECOMBINATION BETWEEN COLK AND PLG500 DETECTED FOLLOWING THE MOBILISATION OF PLG520 OR OF COLK AND PLG500

The donor strains were mated with the recA recipient AB2463 for 30 minutes, as described in chapter 2. The efficiency of mobilisation of Col plasmids was determined as the proportion of R plasmid transconjugants which were immune to colicin K. 50 colicin K immune recipients from each cross were then analysed by 0.5% agarose gel electrophoresis of SDS cleared lysates, to determine whether they had acquired ColK or ColK-pLG500 plasmids.

Donor (GB15)	e.o.m. of Col plasmid	Colicin K immune R Plasmid Transconjugants	
		ColK$^+$	pLG520$^+$
+ R64drd11 + pLG520	60	1	49
+ R64drd11 + ColK + pLG500	96	48	2

VI. Discussion

The experiments described here demonstrate that ColK and pLG500 undergo site-specific, recA independent, reversible recombination. Such recombination, resulting in the cointegration or dissociation of the component replicons, is detected at a frequency of 2-4% amongst colicin K immune R plasmid transconjugants. Since no recombination could be detected in vegetative cultures of donor cells, this suggests either that recombination occurs specifically during transfer, or that it occurs at an undetectably low level during vegetative growth, and the rare recombinant plasmids are only detected if they are subsequently transferred into recipient cells.

Although recombination between ColK and pLG500 was normally detected at a frequency of only 2-4% amongst the colicin K immune R plasmid transconjugants, in the original mating experiment (where ColK-carrying recipients were selected on the basis of immunity to colicin K) 50% of the recipients were found to contain pLG520. On the basis of banding intensity in SDS cleared lysates, pLG520 apparently has a much higher copy number than ColK, and may therefore encode a correspondingly higher level of colicin K immunity. If an inadvertantly high level of colicin K was used to select recipients, this could explain the preponderance of pLG520 plasmids in this experiment. I shall discuss the phenotypic differences between ColK and pLG520 in more detail in a subsequent chapter.

Results presented in this chapter show that the novel type of recombination event which occurs between ColK and pLG500 leads to the formation of composite plasmids which are less relaxable and less efficiently mobilised than ColK. As well as supporting the hypothesis that relaxation proteins are involved in mobilisation, this finding suggests, albeit tentatively, that the recombination event which

generated pLG520 might somehow be associated with the process of relaxation and/or mobilisation of its component replicons. I showed previously that pLG500 requires ColK (or ColE1) coded gene products for its mobilisation and relaxation. As I shall discuss below, in view of the fact that ColK complements pLG500 for mobilisation and relaxation, it is possible to reconcile all of the known features of this recombination phenomenon with the hypothesis that relaxation nicking creates the origin of single-stranded DNA transfer during mobilisation, and that the presumptive nickase-ligase protein of the relaxation complex is subsequently responsible for religating the 3' and 5' ends of the transferred single strands.

It is not yet clear whether pLG500 codes any relaxation proteins, or whether all of these are supplied by ColK. However, since ColK-coded gene products are able to complement the relaxation deficiency of pLG500, relaxation nicking of pLG500 in the presence of ColK should result in the binding of proteins which are at least functionally identical, to the 5' termini of both plasmids. Thereafter, provided the 3' and 5' termini of the two different plasmids were accessible, their single-stranded DNA transfer could result either in recircularisation of each of the transferred plasmid strands (due to intramolecular recognition of 3' ends by the 5' terminally bound protein), or in the formation of ColK-pLG500 composite plasmids (due to intermolecular joining of 3' and 5' ends). Such composite plasmids should be formed recA independently, and the component replicons should always be joined at unique sites (their relaxation nicking sites), in the same relative orientations. Since they would possess hybrid relaxation sites, such plasmids might display altered mobilisation and relaxation properties, but provided these new sites were functional the stable composite plasmids might dissociate to yield the original component replicons when they were themselves mobilised.

It is important to mention that my data does not distinguish whether the recombination event which generates pLG520 plasmids is confined to transfer. In chapter 1 I discussed the possibility that the position of the ColE1 relaxation nicking site with regard to its replication origin might be important in effecting the switch from the normal (Cairns) mode of replication to the transfer (Rolling Circle) mode. Several workers (for example, see Inselburg and Fuke, 1970) have detected a small number of rolling circle molecules amongst normal ColE1 replication intermediates, and it is possible that these molecules arise because there is a low level of spontaneous relaxation nicking even in the absence of a specific mobilisation 'signal'. Therefore, even if pLG520 plasmids are found to be generated in vegetatively growing cells (rather than during transfer), they may well be the products of abortive mobilisation events, and they would then be expected to have identical properties to composite plasmids formed specifically during transfer, as I have described above.

In conclusion, all of the features of pLG520 formation and dissociation are compatible with the hypothesis that such composite plasmids are formed as a consequence of the normal mobilisation process. The aim of the experiments presented in the following chapter was therefore to test some predictions of this hypothesis: namely, whether ColE1, which, like ColK, complements the mobilisation and relaxation deficiency of pLG500, undergoes an analogous recombination event with this miniplasmid, and whether sites of recombination with pLG500 correspond to the ColK and ColE1 bom sites, and relaxation nicking sites.

CHAPTER 7

SITE-SPECIFIC, RECA INDEPENDENT, RECOMBINATION BETWEEN COLE1 AND PLG500: LOCATION OF COLE1 AND COLK MAP POSITIONS AT WHICH RECOMBINATION WITH PLG500 OCCURS

I. Introduction

If the formation of pLG520 plasmids is a consequence of the normal mobilisation process entailing single-stranded DNA transfer from, and strand recircularisation at, relaxation sites, then the component replicons (ColK and pLG500) should be linked together at their relaxation sites. Since both ColE1 and ColK complement the mobilisation and relaxation deficiency of pLG500, a prediction of this hypothesis is that during complementation of the mobility deficiency ColE1-pLG500 plasmids should be generated in an analogous way to pLG520 plasmids.

Since the structure and function of the plasmid ColE1 has been extensively studied a comprehensive functional map of this plasmid is available (for example, see Oka and Takanami, 1976; Dougan et al., 1978), and the map position of the relaxation nicking site is known (Bastia, 1978). If ColE1-pLG500 plasmids (with similar properties to pLG520 plasmids) can be found, it should be possible to locate quite precisely the site in the ColE1 map at which cointegration with pLG500 occurs. Whilst much less is known about the organisation of the ColK genome, a structural and functional analysis of this plasmid is in progress (D. Sherratt, personal communication). Therefore it should also be possible to localise the region of the ColK genome which contains the site for recombination with pLG500.

II. Isolation and Characterisation of ColE1-pLG500 composite plasmids detected following the comobilisation of ColE1 and pLG500

ColE1 and pLG500 were comobilised from the recA donor GB15/R64drd11 + ColE1 + pLG500 to the plasmidless recA recipient AB2463. The efficiency of mobilisation of ColE1, determined as the proportion of R plasmid transconjugants which were colicin E1 immune, was found to be 93%. 50 of these colicin E1 immune recipients were then examined by 0.5% agarose gel electrophoresis of SDS cleared lysates, and 3 of them were thus found to contain a novel plasmid (of 5.2 Mdal) instead of the parental ColE1 plasmid. All of the recipients which were examined by agarose gel electrophoresis were also found to have received pLG500. Of 50 unmated donor colonies which were similarly examined, none appeared to contain these novel plasmids. Some of the colicin E1 immune recipients of the above cross are shown in figure 7.1. One of the plasmids shown is a putative ColE1-pLG500 plasmid, which I have designated pLG540, and it apparently possesses a higher copy number, and is less relaxable, than ColE1.

To determine whether pLG540 was formed by recombination between ColE1 and pLG500, CCC plasmid DNA was prepared, and the HaeII restriction endonuclease digestion products were analysed by 2.0% agarose gel electrophoresis. The results of this analysis, shown in figure 7.2, demonstrate that pLG540 is the product of cointegration of ColE1 and pLG500. Such cointegration involves a site in the ColE1-C and pLG500-A HaeII restriction fragments; since in pLG540 these two fragments are replaced by new fragments of identical total size, recombination must have occurred without the loss or duplication of any ColE1 or pLG500 DNA.

All ColE1-pLG500 plasmids detected following the comobilisation of ColE1 and pLG500, in this and subsequent crosses, were indistinguishable in appearance when examined by 0.5% agarose gel electrophoresis of SDS

Figure 7.1: APPEARANCE OF R PLASMID TRANSCONJUGANTS OF COLE1 AND PLG500 COMOBILISATION

The plasmid content of R plasmid transconjugants of the cross GB15/R64drd11 + ColE1 + pLG500 x AB2463 was examined by 0.5% agarose gel electrophoresis of SDS cleared lysates. Part of a screening gel, showing a recipient that has acquired a ColE1-pLG500 plasmid (slot 5) is shown. Slot 1 shows an SDS cleared lysate of a $ColK^+$ strain. All other slots contain ColE1.

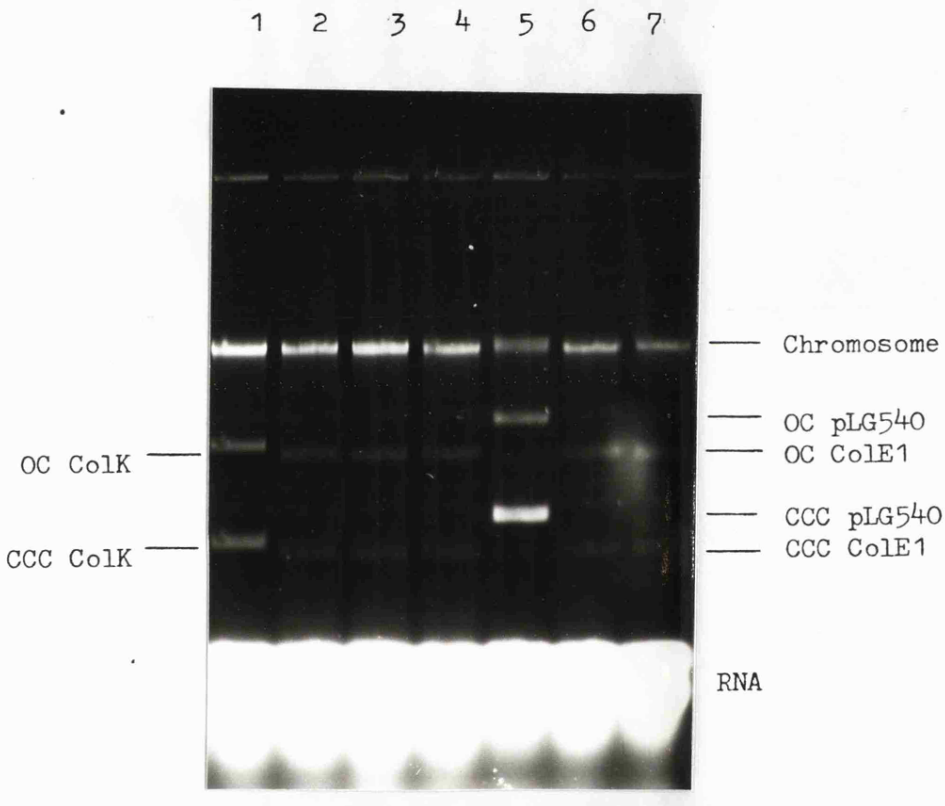

1 2 3 4 5 6 7
OC ColK
CCC ColK
Chromosome
OC pLG540
OC ColE1
CCC pLG540
CCC ColE1
RNA

Figure 7.2: HaeII RESTRICTION ANALYSIS OF COLE1, PLG500, AND PLG540

Supercoiled plasmid DNA was prepared from GB15/R64drd11 + ColE1 + pLG500 and from GB15/R64drd11 + pLG540 + pLG500 by CsCl-EtBr gradient centrifugation, and the different supercoiled plasmid DNA species were separated by preparative neutral sucrose gradient centrifugation. The purified plasmid DNA preparations were then digested with HaeII restriction endonuclease, and analysed by 2.0% agarose gel electrophoresis.

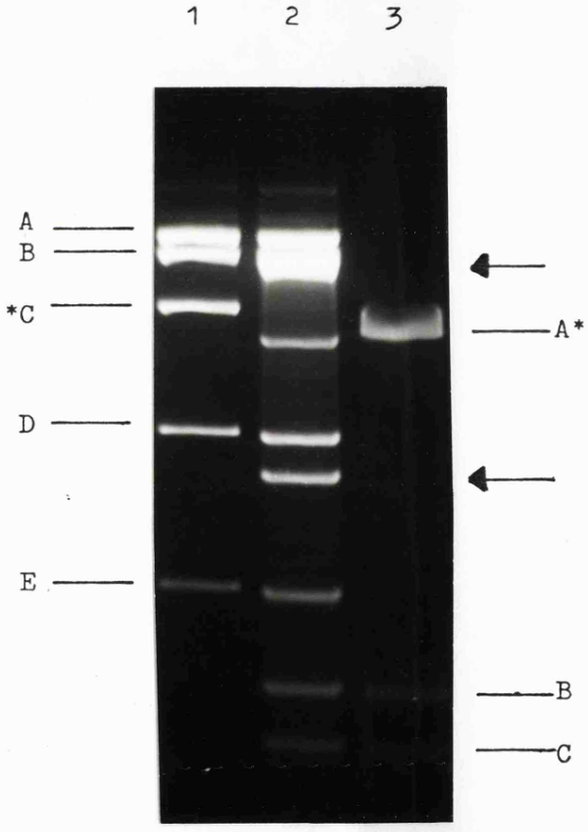
slot 1: ColE1 x HaeII

slot 2: pLG540 x HaeII

slot 3: pLG500 x HaeII

ColE1 and pLG500 restriction fragments which are absent from pLG540 are marked by an asterisk. The novel pLG540 fragments are indicated by arrows.

The pLG540 DNA preparation also contains some pLG500 DNA, and this explains why a restriction fragment which comigrates with the HaeII-A fragment of pLG500 can be seen in slot 2.


1
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3
A
B
*C
D
E
A*
B
C

cleared lysates. These composite plasmids were all less relaxable, and apparently possessed a higher copy number, than ColE1. Therefore the possibility that they were formed by site-specific recombination between ColE1 and pLG500 was investigated. CCC plasmid DNA was prepared from five independently isolated recipients of ColE1-pLG500 plasmids, and subjected to HaeII restriction analysis. In each case the restriction pattern was found to be identical to that of the original pLG540 plasmid shown in figure 7.2, indicating that these plasmids were formed by recombination between unique sites in the ColE1 and pLG500 genomes.

Isogenic recA donor strains carrying R64drd11 and pLG540, or ColE1 and pLG500, were constructed, and matings with a recA recipient (AB2463) were performed in order to compare the efficiency of mobilisation of the Col plasmids, and to determine whether the component replicons of pLG540 dissociated as did those of pLG520. Mobilisation frequencies of Col plasmids were determined as the proportion of R plasmid transconjugants which acquired immunity to colicin E1. 50 colicin E1 immune recipients of each cross, and 50 unmated donor colonies, were examined by 0.5% agarose gel electrophoresis of SDS cleared lysates. The results of these matings are summarised in table 7.1. As indicated, some colicin E1 immune recipients of pLG540 mobilisation received ColE1 instead of pLG540. (The plasmid was ajudged to be the original ColE1 plasmid because it was identical to ColE1 in size and appearance; in one case its identity was confirmed by HaeII restriction analysis). Therefore the recombination event which generates pLG540 plasmids is reversible. These results also show that the mobilisation frequency of pLG540 is significantly lower than that of ColE1.

Table 7.1

FREQUENCY OF RECOMBINATION BETWEEN COLE1 AND PLG500 DETECTED FOLLOWING THE MOBILISATION OF PLG540 OR OF COLE1 AND PLG500

The donor strains were mated with the recA recipient AB2463 for 30 minutes, as described in chapter 2. The efficiency of mobilisation of Col plasmids was determined as the proportion of R plasmid transconjugants which were immune to colicin E1. 50 colicin E1 immune recipients, and 50 unmated donor colonies, from each cross were examined by 0.5% agarose gel electrophoresis of SDS cleared lysates to determine whether they contained ColE1 or ColE1-pLG500 plasmids. All donor colonies were found to be identical in appearance, and ColE1, but not ColE1-pLG500, plasmids were visible in each case.

Donor (GB15)	e.o.m. of Col Plasmid	Colicin E1 immune R Plasmid Transconjugants	
		ColE1$^+$	pLG540$^+$
+ R64<u>drd</u>11 + pLG540	26%	2	48
+ R64<u>drd</u>11 + ColE1 + pLG500	95%	46	4

III. Location of the Site in the ColE1 Genome at which Recombination with pLG500 occurs

Since ColE1-pLG500 plasmids, with analogous properties to ColK-pLG500 plasmids, could be isolated, an attempt was made to locate the exact site in the ColE1 genome at which cointegration with pLG500 occurred. In figure 7.3 a functional map of ColE1, showing HaeII and HaeIII restriction sites, is presented. This map is based largely on the data of Oka and Takanami (1976), and Dougan et al. (1978). The HaeII fragment of ColE1 which contains the site of cointegration with pLG500 has already been identified as the HaeII-C fragment (see figure 7.2). HaeIII restriction analysis of ColE1 and pLG540 is presented in figure 7.4. pLG500, not shown here, has a single HaeII site (for example, see figure 4.3). This analysis demonstrates that the site for cointegration with pLG500 is within one of two unresolvable restriction fragments - the HaeIII-E and HaeIII-F fragments. Since the HaeIII-E fragment of ColE1 overlaps with the HaeII-C fragment, but the HaeIII-F fragment is not in this region, the recombination site must lie in the HaeIII-E - HaeII-C overlap region. This part of the genome, which constitutes about 350 base pairs, appears to be an intercistronic region (see figure 7.3), and it is known to contain the relaxation site (Bastia, 1978).

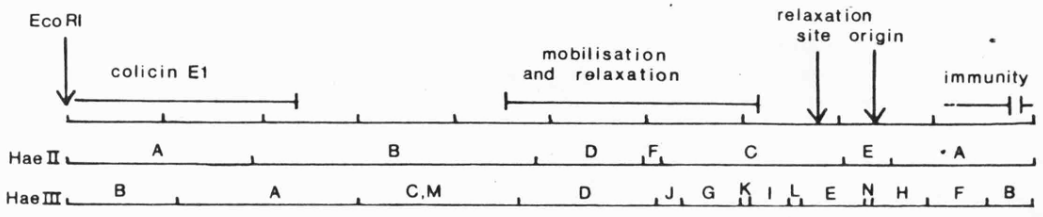
The region surrounding the ColE1 relaxation site has been sequenced (Bastia, 1978), and recently Oka et al. (1979) have determined the entire nucleotide sequence of a small ColE1 derivative which constitutes one quarter of the ColE1 genome and contains the ColE1 replication origin and relaxation nicking site. Figure 7.3 includes a fine structure map of the region spanning the relaxation site, and shows the position of relevant HaeII, HaeIII, HhaI, and HpaII restriction sites, deduced from the data of Oka et al. The results of HhaI and HpaII restriction analysis of pLG540 and its component replicons are shown in figure 7.5.

Figure 7.3: A MAP OF PLASMID COLE1, SHOWING THE REGION SURROUNDING THE RELAXATION SITE AND REPLICATION ORIGIN

(a) A functional map of ColE1, showing HaeII and HaeIII restriction sites, based largely on data of Oka and Takanami (1976), and Dougan et al. (1978).

(b) The region surrounding the relaxation nicking site and replication origin, showing the position of relevant HaeII, HaeIII, HpaII, and HhaI restriction sites (see text). The map is based largely on sequencing data of Oka et al. (1979).

(a)


EcoRI
colicin E1
mobilisation and relaxation
relaxation site
origin
immunity
Hae II
A
B
D
F
C
E
A
Hae III
B
A
C,M
D
J
G
K
I
L
E
N
H
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(b)

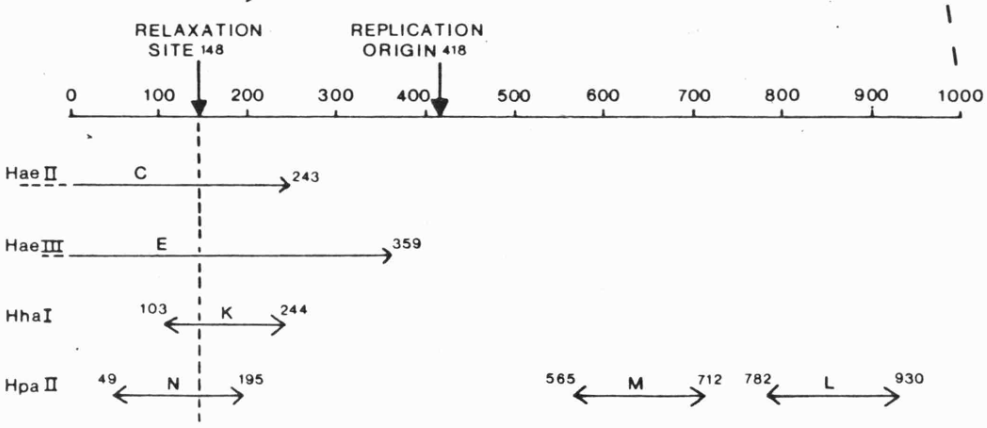
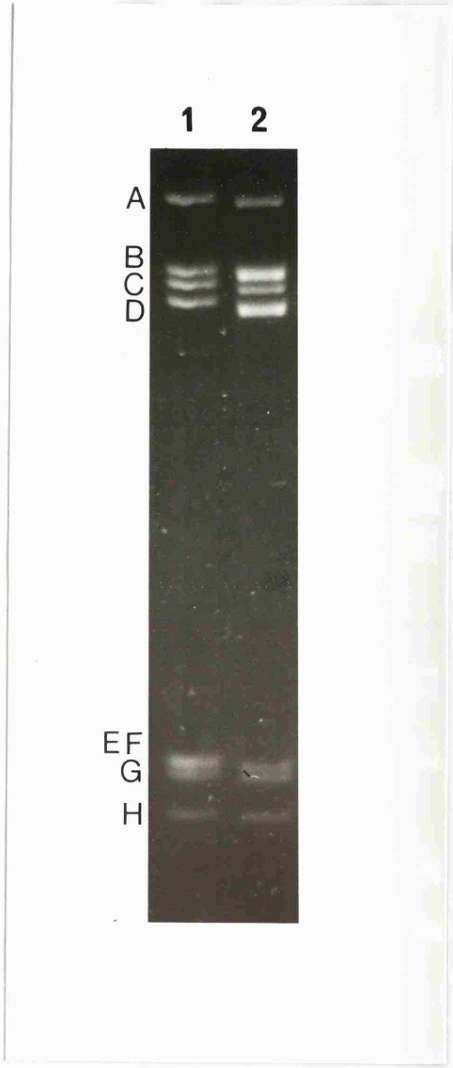

RELAXATION SITE 148
REPLICATION ORIGIN 418
0
100
200
300
400
500
600
700
800
900
1000
Hae II
C
243
Hae III
E
359
Hha I
103
K
244
Hpa II
49
N
195
565
M
712
782
L
930

Figure 7.4: HaeIII RESTRICTION ANALYSIS OF COLE1 AND PLG540

Purified plasmid DNA samples were digested with HaeIII restriction endonuclease and then subjected to 2.5% agarose gel electrophoresis for 10 hours at 100 volts. pLG500 which is not included here has a single HaeIII site.

slot 1: ColE1 x HaeIII

slot 2: pLG540 x HaeIII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D
E F
G
H

Figure 7.5: HhaI AND HpaII RESTRICTION ANALYSIS OF PLG540 AND ITS COMPONENT REPLICONS

Purified plasmid DNA species were digested with HhaI or HpaII restriction endonculeases, and then subjected to 4.0% agarose gel electrophoresis for 18 hours at 50 volts. A HaeIII digest of ColE1 was included as molecular weight markers.

slot 1: ColE1 x HhaI

slot 2: pLG540 x HhaI

slot 3: pLG500 x HhaI

slot 4: ColE1 x HpaII

slot 5: pLG540 x HpaII

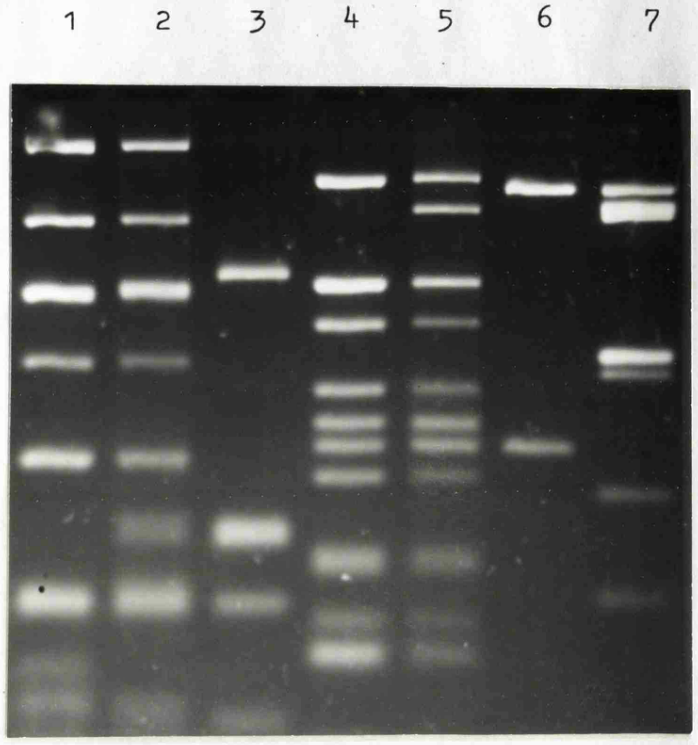
slot 6: pLG500 x HpaII

slot 7: ColE1 x HaeIII

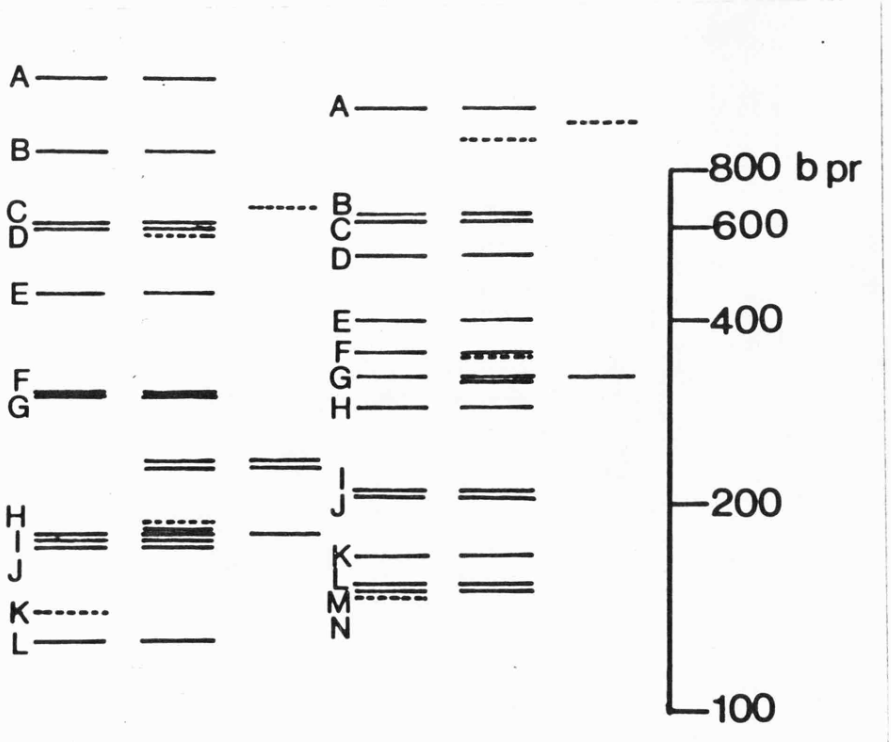
(a) Photograph of the gel.

(b) A line drawing of the results. Restriction fragments containing the sites for recombination are drawn as dotted lines. A scale indicating the size of fragments is included on the right hand side of the figure.

(a)


1
2
3
4
5
6
7

(b)


A
B
C
D
E
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J
K
L
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E
F
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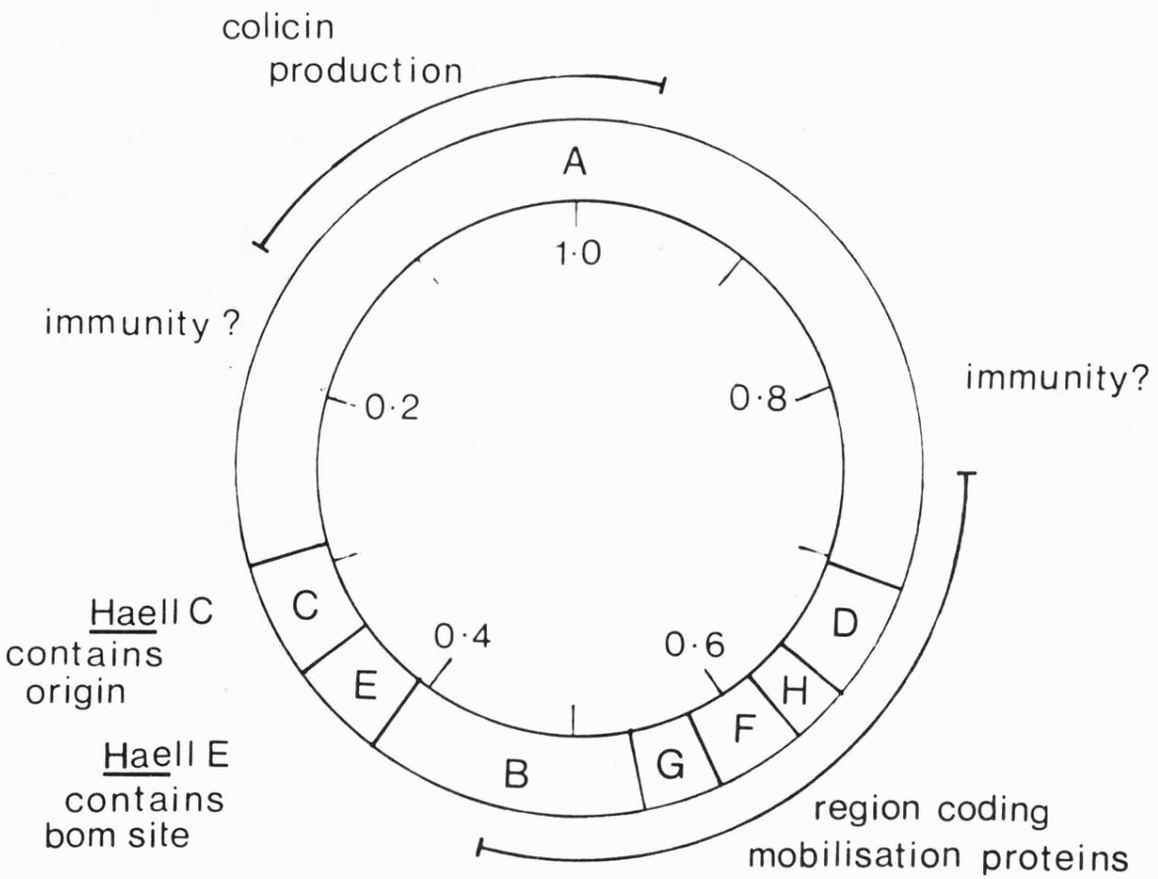
H
I
J
K
L
M
N
800 b pr
600
400
200
100

Figure 7.6: A FUNCTIONAL MAP OF PLASMID COLK

Regions involved in Colicin K production and immunity, and plasmid replication and mobilisation, are indicated on the HaeII map of plasmid ColK, communicated by D. Sherratt.


colicin
production
A
1·0
immunity ?
0·2
0·8
immunity?
C
E
0·4
0·6
D
H
F
G
B
HaeII C
contains
origin
HaeII E
contains
bom site
region coding
mobilisation proteins

HhaI restriction analysis shows that the site for recombination lies within the HhaI-K fragment, and Bastia (1978) has reported that this fragment spans the relaxation site. HpaII restriction analysis shows that the site for recombination is within one of three unresolvable (L, M, or N) fragments. Three HpaII fragments of the appropriate size (148, 147, and 146 base pairs) are all located within the small region shown in figure 7.3. However, only one of these - the HpaII-N fragment - is also contained within the HaeIII-E restriction fragment. The site for recombination must therefore lie within the region common to the HaeII-C, HaeIII-E, HhaI-K, and HpaII-N restriction fragments. This localises it to within a 92 base pair sequence (within bases numbered 103-195 in figure 7.3). This region spans the ColE1 relaxation site, which is located at base 148. Therefore the site at which ColE1 and pLG500 recombine must lie within 47 base pairs of the ColE1 relaxation nicking site.

IV. Location of the Site in the ColK Genome at which Recombination with pLG500 occurs

In a current study deletion and insertion mutants of ColK have been isolated, and this has enabled the regions of the genome involved in colicin production, colicin immunity, autonomous replication, and conjugal mobility to be localised (D. Sherratt, personal communication). A functional map of ColK, showing HaeII restriction sites, has been communicated by D. Sherratt, and is shown in fugure 7.6. The genetically defined transfer origin (or bom site) of ColK lies within the HaeII-E fragment. HaeII restriction analysis of pLG520 and its component replicons has already been presented (see figure 6.1), and this shows that the site for cointegration with pLG500 lies in the HaeII-E fragment. Therefore the site of cointegration with pLG500 is within the same

(350 base pair) region of ColK as is known to contain sequences essential for ColK mobilisation.

V. RP4-Promoted Comobilisation of ColE1 and pLG500

To determine whether pLG540 (and pLG520) plasmids were only formed in the presence of the sex factor R64drd11, the ability of a different conjugative plasmid - RP4 - to promote the comobilisation of ColE1 and pLG500 was examined. As I shall elaborate later, there were several reasons for choosing RP4 as an alternative sex factor.

The recA strain GB2/RP4 + ColE1 + pLG500 was constructed, and used as a donor in a 30 minute mating with the recA recipient GB15. The efficiency of mobilisation of ColE1, determined as the proportion of RP4-carrying transconjugants that were colicin E1 immune, was found to be 20%. To determine whether pLG500 had been mobilised, R plasmid transconjugants were examined by 1.2% agarose gel electrophoresis of RNAase treated SDS cleared lysates. In this way 50% of the R plasmid transconjugants were found to have received pLG500. It was clear from the gel showing these recipients that the extent of relaxability of ColE1 and pLG500 plasmids was similar in the presence of RP4 to that observed in the presence of R64drd11 (data not shown).

When 50 colicin E1 immune transconjugants were examined by agarose gel electrophoresis of SDS cleared lysates, one was found to have received a plasmid identical in size and appearance to pLG540 plasmids, instead of ColE1. Supercoiled putative ColE1-pLG500 plasmid DNA was prepared from this recipient and its HaeII restriction pattern compared to that of pLG540. Agarose gel electrophoresis of the digestion products confirmed that this plasmid was identical to pLG540.

VI. Discussion

Results presented in this chapter demonstrate that ColE1, like ColK, undergoes site-specific, *recA* independent, reversible recombination with pLG500, which can be detected following R64*drd*11 promoted comobilisation of these plasmids. For both ColE1 and ColK, genetic analysis of deletion mutants has recently enabled the sequences essential for their mobilisation to be localised to within unique regions of three to four hundred base pairs. Restriction analysis of pLG540 and pLG520 plasmids presented here demonstrates that the sites in the ColE1 and ColK genomes at which recombination with pLG500 occurs lie within the same regions. Moreover, in the case of ColE1, where the region containing the *bom* site has been sequenced, I have shown that the site at which recombination with pLG500 occurs must lie within 47 base pairs of the relaxation nicking site.

As discussed in the previous chapter, all these observations are consistent with the hypothesis that pLG540 and pLG520 plasmids are formed as a consequence of the process of plasmid mobilisation by single-stranded DNA transfer from, and strand recircularisation at, relaxation nicking sites. Although I was not able to determine the exact base pair in ColE1 at which recombination with pLG500 occurs, I have localised it to within a 92 base pair intercistronic region which includes the relaxation nicking site. Since a specific DNA nicking reaction can be demonstrated at the ColE1 relaxation site, this site is a very strong candidate for the recombination site. In the case of ColK a relaxation complex has never been isolated, yet it is clear (see chapter 5) that ColK does code proteins which can facilitate relaxation nicking of pLG500 plasmid DNA. Therefore the finding that for both ColE1 and ColK plasmids the recombination sites map in the same regions as the genetically defined transfer origins strongly suggests that such sites

correspond to the recombination sites, and that these are also the sites for relaxation nicking and strand recircularisation following transfer.

A scheme for plasmid mobilisation, which accounts for pLG540 plasmid formation as a consequence of ColE1 complementing the mobilisation and relaxation deficiency of pLG500, is presented in figure 7.7. According to this model ColE1 relaxation proteins recognise DNA sequences at or near the relaxation sites of both ColE1 and pLG500, and during conjugation they nick at the relaxation sites to create the origins of single-stranded DNA transfer. Relaxation nicking is accompanied by the binding of the same species of ColE1 relaxation protein (the presumptive nickase-ligase) to the 5' ends of the relaxed strands of both plasmids. Relaxed single strands of ColE1 and pLG500 plasmid DNA are then transferred, probably via a leading 5' terminus, and ligation of 3' and 5' ends is mediated by the protein bound to the 5' terminus. Since the same (or at least functionally identical) protein species is bound to the 5' end of both ColE1 and pLG500, 3' ends of the different plasmids are not discriminated, and therefore intermolecular ligation, as well as intramolecular ligation, of the transferred strands can occur.

In conclusion, all the features of pLG540 and pLG520 formation that I have described suggest that these plasmids are formed as the consequence of single-stranded DNA transfer from, and strand recircularisation at, a unique site, probably the relaxation nicking site. Until these plasmids were discovered and characterised, the events which occurred during plasmid mobilisation (after relaxation nicking) were almost totally unexplored. Only one relevant observation had been made, by Inselburg and Ware (1979), who found that mutants of ColE1 which were Mob$^-$ but still relaxable could be isolated, suggesting a role for ColE1-coded gene products in mobilisation after relaxation nicking had occurred. As I shall discuss in detail in the final chapter, and as

Figure 7.7: SCHEME FOR PLG540 PLASMID FORMATION DURING THE COMOBILISATION OF COLE1 AND PLG500

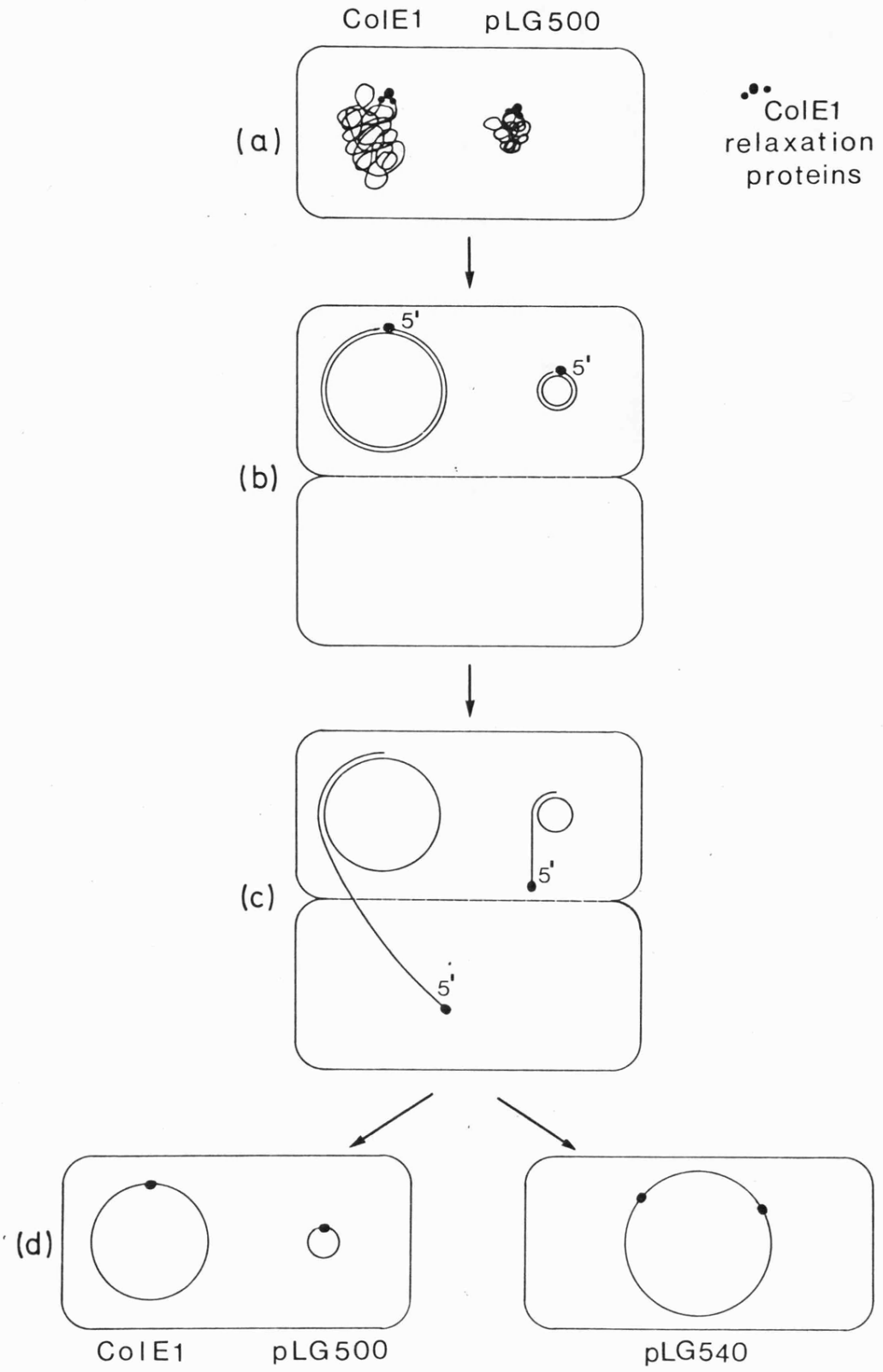
Only pre-existing DNA strands are shown as nothing is known about complementary strand synthesis, and the timing of it would not affect the model. Here pLG500 is assumed to code none of its own relaxation proteins; but even if it codes some, functionally identical protein species would bind to the 5' termini of both plasmids (part b), thus the outcome of the model would be unaffected.

(a) Proteins of the ColE1 relaxation complex bind at or near the relaxation nicking sites of both ColE1 and pLG500.

(b) Prior to transfer, the ColE1 relaxation proteins produce a single-stranded nick at the relaxation sites of both ColE1 and pLG500, and one of the proteins (the presumptive nickase-ligase) covalently binds to the exposed 5' terminal nucleotides of both relaxed molecules.

(c) Relaxed single strands of ColE1 and pLG500 DNA, with identical proteins attached to their leading 5' ends, are transferred to recipient cells.

(d) The proteins bound to the 5' ends recognise the 3' plasmid termini: this normally involves single molecules and therefore results in the recircularisation of transferred plasmid molecules. But since the 5' termini of both ColE1 and pLG500 plasmids possess identical proteins, intermolecular ligation can occur, resulting in the formation of composite (pLG540) plasmids.


ColE1
pLG500
(a)
ColE1
relaxation
proteins
5'
5'
(b)
5'
5'
(c)
(d)
ColE1
pLG500
pLG540

results presented here already imply, it seems very likely that the study of such composite plasmids can provide a valuable insight into the process of plasmid mobilisation.

Since pLG540 plasmids can be detected following the comobilisation of ColE1 and pLG500 by the conjugative plasmid RP4, this demonstrates that such recombination is not peculiar to strains carrying R64drd11. It is known that the plasmids R751 and R388 can promote the mobilisation of wild type ColE1, but not of a derivative containing only the sequences essential for mobilisation by R64drd11 (Warren et al., 1979). This finding raises the question of whether multiple mechanisms for ColE1 mobilisation (requiring additional regions of the ColE1 genome) exist, or whether the same basic mechanism always operates, but extra ColE1-coded gene products are sometimes necessary if the conjugative plasmid is to be able to promote mobilisation. Since RP4 falls into the same category as R751 and R388 plasmids (D. Sherratt, personal communication), and since pLG540 plasmids, which are thought to be the products of the normal mobilisation process, were detected following RP4 promoted mobilisation of ColE1 and pLG500, this implies that the same basic mechanism is responsible for mobilisation during R64drd11 and RP4 mediated transfer.

Whilst the efficiency of mobilisation of ColE1 and pLG500 is lower during RP4 promoted mobilisation than during R64drd11 promoted mobilisation, it is interesting to note that pLG500 is mobilised more than twice as efficiently as ColE1. During R64drd11 promoted mobilisation of ColE1 and pLG500, since both small plasmids were very efficiently mobilised it was impossible to tell whether pLG500 was mobilised at a significantly greater frequency than pLG500. Here however, where some factor other than the availability of relaxation proteins seems to be limiting, it is clear that pLG500 is mobilised

much more efficiently than ColE1.

CHAPTER 8

A COMPARISON OF THE PROPERTIES OF PLG520 AND PLG540 WITH THOSE OF THEIR COMPONENT REPLICONS - PLG500, COLK, AND COLE1

I. Introduction

Results presented in the last two chapters show that whilst pLG520 and pLG540 plasmids resemble ColK and ColE1 in that they produce, and are immune to, the appropriate colicins, they are strikingly different to their component Col plasmids in terms of copy number, and in their mobilisation and relaxation properties. In this chapter I shall compare the properties, and analyse the interactions, of these composite plasmids with their component replicons. Since pLG520 and pLG540 plasmids are only detected following mobilisation, yet their properties may well influence the outcome of the mobilisation experiments, this analysis may help to provide an important new perspective on the recombination process.

II. Copy Number

The results of agarose gel electrophoresis of SDS cleared lysates of ColK and pLG520, and ColE1 and pLG540, were shown in figures 6.2 and 7.1 respectively. In both cases the composite plasmids appear to have a higher copy number (and to be less relaxable) than the ColK and ColE1 plasmids. In table 8.1 the results of figures 6.2 and 7.1 are presented semi-quantitatively: microdensitometry tracings of gel tracks were made, and the intensity of each plasmid band relative to that of the chromosomal band was recorded.

Whilst detailed comparisons of the same plasmid isolated from cells

Table 8.1

BANDING INTENSITY OF CCC AND OC FORMS OF COLK AND PLG520, AND COLE1 AND PLG540, PLASMID DNA IN SDS CLEARED LYSATES

The intensity of banding of ColK, pLG520, ColE1, and pLG540 plasmid DNA species in SDS cleared lysates (figures 6.2 and 7.1) was measured by microdensitometry tracings of gel tracks. The relative amounts of CCC and OC plasmid DNA compared to chromosomal DNA (1.0 arbitrary units) is recorded.

Plasmid	Chromosome	CCC	OC	CCC/OC
ColK	1.0	0.33	0.26	1.2
pLG520	1.0	3.2	1.4	2.3
ColE1	1.0	0.14	0.18	0.8
pLG540	1.0	2.6	0.72	3.6

grown under different conditions can be made by agarose gel electrophoresis of SDS cleared lysates, several factors must be considered when different plasmids are being compared. Firstly, it is not known whether during a clearing spin different plasmids (even if they are similar in size) are recovered to the same extent. Secondly, the intensity of plasmid DNA banding will depend both on the size and configuration of the plasmid, therefore hindering comparisons between plasmids of different sizes, with different proportions of CCC and OC DNA. However even if these factors have influenced the results shown in table 8.1, the very large difference in the proportions of pLG520 and pLG540 plasmid DNA, compared to ColK and ColE1 DNA, strongly suggests that there is a large difference in the copy number of the composite plasmids compared to the original Col plasmids.

To circumvent the potential problems associated with a clearing spin, an attempt to compare the copy numbers of ColK and pLG520 was made by examining the amounts of plasmid DNA present in whole cell lysates. 25 ml L broth grown, mid log phase cultures, of three different strains carrying ColK or pLG520 were harvested at an identical optical density (A_{600} = 0.4), and SDS-lysed in a total volume of 300 µl. Each sample was vortexed for 10 seconds, thus enabling 30 µl aliquots to be pipetted and loaded into the slots of an agarose gel. The samples were subjected to 0.5% agarose gel electrophoresis, for 15 hours, at 30 volts. The results of this experiment are shown in figure 8.1, and presented semi-quantitatively in table 8.2. Under these electrophoresis conditions the large chromosomal DNA fragments do not migrate very far into the gel, and bands of CCC and OC plasmid DNA are visible below the chromosomal DNA. The data summarised in table 8.2 indicates that there is substantially more CCC pLG520 DNA than CCC ColK DNA, whilst the amounts of OC DNA are similar. Unless the increased intensity of

Figure 8.1: COMPARATIVE LEVELS OF COLK AND PLG520 PLASMID DNA

Three isogenic strains carrying either ColK or pLG520 were used to prepare whole cell lysates as described in the text. Identical volumes of each lysate were subjected to 0.5% agarose gel electrophoresis at 30 volts overnight.

slot 1: C600/R64drd11 + pLG520

slot 2: C600/R64drd11 + ColK

slot 3: JB2/R64drd11 + pLG520

slot 4: JB2/R64drd11 + ColK

slot 5: GB15/R64drd11 + pLG520

slot 6: GB15/R64drd11 + ColK

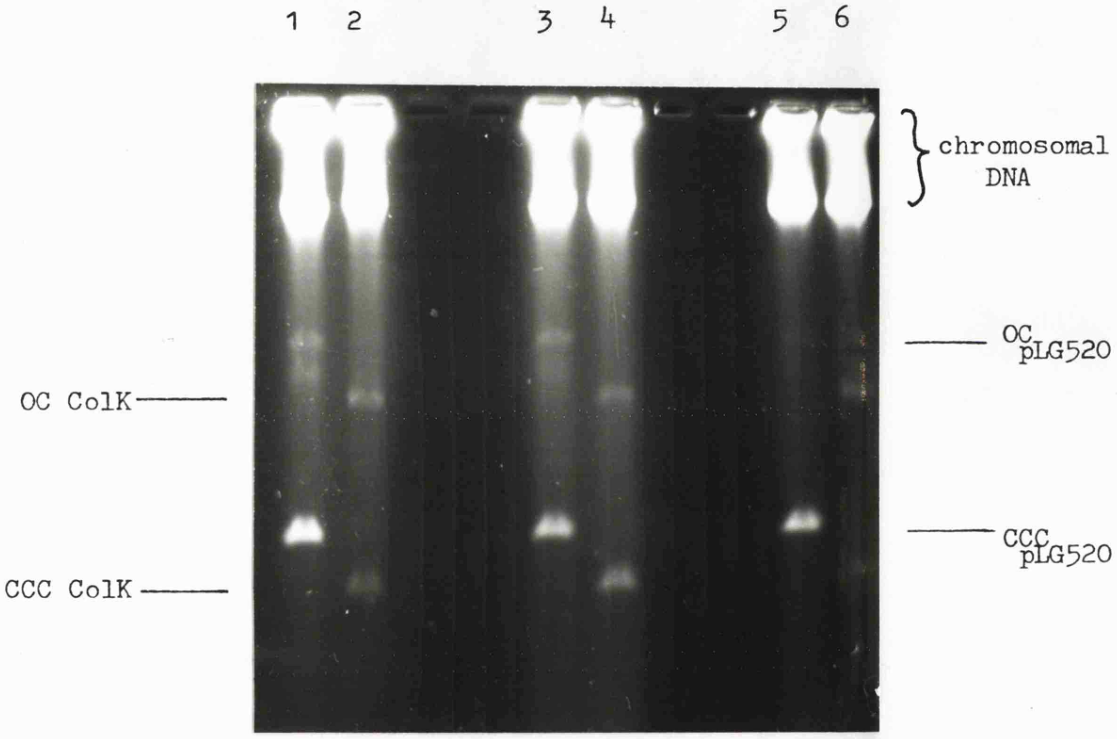

1 2 3 4 5 6
chromosomal DNA
OC pLG520
CCC pLG520
OC ColK
CCC ColK

Table 8.2

INTENSITY OF BANDING OF THE CCC AND OC FORMS OF COLK AND PLG520 IN SDS WHOLE CELL LYSATES

The intensity of banding of CCC and OC plasmid DNA species was measured by microdensitometry tracing of the gel tracks of figure 8.1. The absolute amounts of CCC and OC plasmid DNA are recorded in arbitrary units.

Strain	CCC ColK	OC ColK	CCC pLG520	OC pLG520	$\frac{\text{CCC pLG520}}{\text{CCC ColK}}$	$\frac{\text{CCC ColK}}{\text{OC ColK}}$	$\frac{\text{CCC pLG520}}{\text{OC pLG520}}$
JB1	5	5	17	6	3.4	1.0	2.8
JB2	3	3	15	2.5	5.0	1.0	6.0
GB15	7	3	13.5	2.5	1.9	2.3	5.4

banding of the CCC pLG520 plasmid DNA can be attributed to its increased molecular weight, or the ColK plasmid is preferentially trapped amongst the chromosomal DNA fragments, this experiment indicates that pLG520 has a significantly higher copy number than ColK.

No attempt was made to measure the copy number of the miniplasmid pLG500. However, since the intensity of banding of plasmid DNA in agarose gels is size dependent, the fact that pLG500 was clearly visible in SDS cleared lysates indicates that this plasmid must have a high copy number.

III. Colicin Production by ColK and pLG520

Since an increase in copy number can often be correlated with the increased production of a plasmid-coded gene product, levels of colicin K production in cells carrying ColK or pLG520 were compared. L broth overnights of the Rec$^+$ strain JB2/R64drd11 + ColK, and of JB2/R64drd11 + pLG520, were diluted to the same optical density, and 5 µl, or 15 µl, aliquots of both cultures were spotted onto nutrient agar plates. After overnight incubation of these plates the bacterial growth was killed by chloroforming, and then the plates were overlaid with soft nutrient agar seeded with 10^7 cells of a colicin K sensitive strain (5K). The appearance of these plates after a further overnight incubation is shown in figure 8.2. Since the zone of killing of the colicin K sensitive lawn is much larger around the pLG520$^+$ cells than around the ColK$^+$ cells, this demonstrates that the composite plasmid encodes much higher levels of colicin K production than its component replicon, ColK.

When the above experiment was repeated using the strain JB2/R64drd11 + pLG500, no killing of the colicin K sensitive lawn could be detected, thus confirming that pLG500 is a non-colicinogenic plasmid.

Figure 8.2: LEVELS OF COLICIN K PRODUCTION ENCODED BY COLK AND PLG520

Identical volumes of JB2/R64drd11 + ColK and JB2/R64drd11 + pLG520 were spotted, in duplicate, onto nutrient agar plates. The overnight bacterial growth was killed by chloroforming, and the dead cells were overlaid with a colicin K sensitive indicator strain (5K). The appearance of plates after a further overnight incubation at 37°C is shown. Aliquots of the pLG520-carrying strain were spotted onto the top half of plates, and the ColK-carrying strain was spotted onto the bottom half.

(a) 15 µl aliquots of cultures, A_{600}= 0.2, were spotted.

(b) 5 µl aliquots of cultures, A_{600}= 0.2, were spotted.

(a)

(b)

IV. Mobilisation Frequencies and Levels of SDS-Induced Relaxation

The experiments described in chapters 6 and 7 show that ColK and ColE1 plasmids are mobilised at a frequency of 90-95% during R64drd11 mediated conjugation. However, in similar experiments the composite plasmids pLG520 and pLG540 were found to be less efficiently mobilised: values of 55-70% were found for pLG520, and of only 20-30% for pLG540. The data summarised in tables 8.1 and 8.2 indicate that, following SDS-induced relaxation nicking, the proportion of plasmid DNA in the OC form is higher for ColK and ColE1 than for pLG520 and pLG540 plasmids. Therefore the composite plasmids derived from ColK and ColE1 are both less relaxable and less efficiently mobilised than the parental Col plasmids.

In chapter 5 I showed that pLG500 (in the presence of R64drd11 alone) was an unrelaxable, non-mobilisable, plasmid, yet it could be rendered relaxable and mobilisable in the presence of ColK or ColE1. When mobilisation experiments were performed using the plasmid RP4, rather than R64drd11, to promote conjugation, it became clear that complementation of the pLG500 mobility defect by ColE1 resulted in pLG500 being mobilised at a significantly higher frequency than ColE1 itself. Previously it was noted (see chapter 5, section V) that complementation of the relaxation deficiency of pLG500 by ColE1 or ColK apparently resulted in a higher level of relaxation of the miniplasmid than the complementing plasmid. Therefore, although pLG500 requires ColE1 (or ColK) coded gene products for its mobilisation and relaxation, provision of these gene products results in higher levels of mobilisation and relaxation for pLG500 than for the complementing plasmid.

V. Trans Interactions between pLG540 and its Component Replicons

When the recipients of comobilisation of ColE1 and pLG500 were initially screened, those which had acquired pLG540 (ColE1-pLG500) plasmids were also found to contain pLG500. Similarly, recipients of comobilisation of ColK and pLG500 that had acquired pLG520 (ColK-pLG500) plasmids were also found to contain pLG500 (but never ColK). In the few cases so far studied it has always been found that naturally occurring plasmids are incompatible with self-derivatives made by inserting extra DNA. Therefore it would be interesting to see whether pLG500 was really compatible with pLG540 (or whether its persistence in cells simply reflected its high copy number, and in future generations it would be gradually diluted from the population due to incompatibility). Since recipients of pLG540 (or pLG520) plasmids never contained ColE1 (or ColK), this suggested that the composite plasmids were incompatible with the Col plasmids from which they were derived. Clearly, if such incompatibility existed, this would influence the final outcome of the comobilisation experiments following which composite plasmids were detected. Therefore experiments were performed to examine the incompatibility relationships between pLG540 and its component replicons.

Since ColE1 and pLG540 plasmids cannot be easily distinguished phenotypically, the incompatibility relationship between pLG540 and an ampicillin resistant, non-colicin producing, derivative of ColE1 (ColE1-Ap) was examined. ColE1-Ap consists of the single EcoRI fragment of ColE1 ligated to an EcoRI fragment which carries the genes for ampicillin resistance. This ampicillin resistance fragment is derived from the Staphylococcus aureus plasmid pI258 (see Peyru, Wexler, and Novick, 1969; Timmis, Cabello, and Cohen, 1975).

The donor strain GB15/R64drd11 + pLG540 was mated with the

recipients JB1, and JB1/ColE1-Ap, for 1 hour. R plasmid transconjugants were then selected, and 100 colonies from each cross were examined for colicin production and ampicillin resistance. The results of this analysis, which are shown in table 8.3, indicate that pLG540 is mobilised slightly less efficiently into JB1/ColE1-Ap than into JB1, but that most recipients which become colicin-producing (24 out of 32) lose the ColE1-Ap plasmid. Three of the recipients which were found to be both Col^+ and ampicillin resistant were then restreaked to single colonies, and four colonies from each purification were again examined for ampicillin resistance and colicin production. All of the colonies tested were found to be ampicillin-sensitive and colicin-producing. These results demonstrate that pLG540 is incompatible with ColE1-Ap, and that ColE1-Ap is displaced from cells by an incoming pLG540 plasmid.

To determine whether pLG500 and pLG540 were incompatible a transconjugant which had received both these plasmids (GB15/R64drd11 + pLG540 + pLG500) was grown in L broth for approximately 40 generations, and then analysed as follows. The culture was streaked out to single colonies on a nutrient agar plate containing 100 µg/ml spectinomycin. Five colonies were then chosen at random, restreaked across a spectinomycin plate, and used to make SDS cleared lysates. Each lysate was RNAase treated and subjected to 1.2% agarose gel electrophoresis in order to determine whether pLG500 was still present. In this way all of the lysates examined were found to be identical in appearance, both with one another and with the original transconjugant. The appearance of one of these lysates, and of similarly prepared SDS cleared lysates of GB15/R64drd11 + ColE1 + pLG500, and GB15/R64drd11 + pLG500, is shown in figure 8.3. Since pLG500 was still present, 40 generations after first being detected, in each of the five colonies examined, this indicates either that pLG500 is compatible with pLG540, or that it

Table 8.3

INCOMPATIBILITY BETWEEN PLG540 AND COLE1-AP

The donor GB15/R64drd11 + pLG540 was mated with the recipients JB1 and JB1/ColE1-Ap, for 1 hour. R plasmid transconjugants were selected, and 100 from each cross were examined for colicin production and ampicillin resistance.

Recipient	R Plasmid Transconjugants			
	$Col^+ Ap^r$	$Col^+ Ap^s$	$Col^- Ap^r$	$Col^- Ap^s$
JB1	—	42	—	58
JB1/ColE1-Ap	8	24	68	0

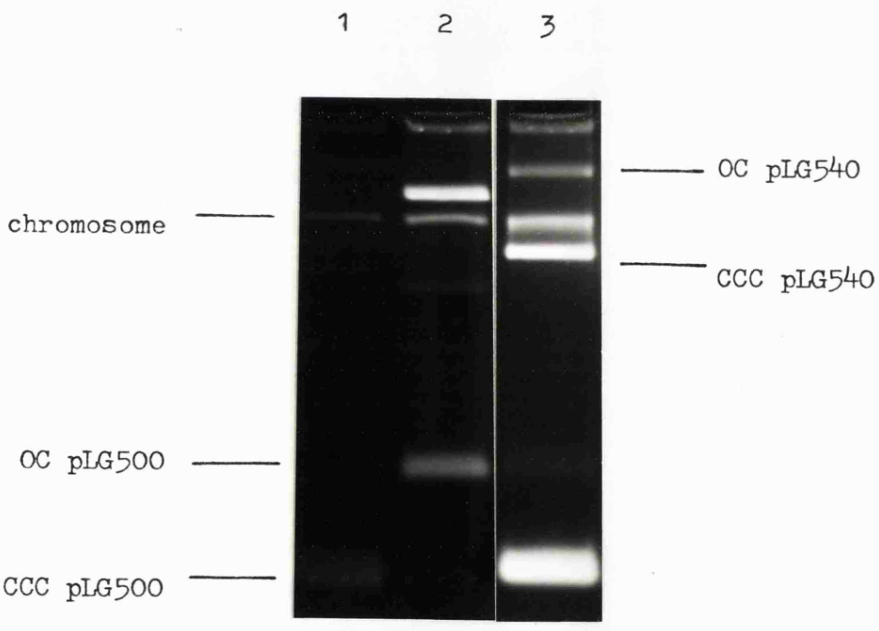
Figure 8.3: TRANS EFFECT OF PLG540 ON PLG500

RNAase treated cleared lysates of pLG500-carrying strains, including a recipient of pLG540 and pLG500, were subjected to 1.2% agarose gel electrophoresis so that the conformation of the pLG500 plasmid could be examined.

slot 1: GB15/R64drd11 + pLG500

slot 2: GB15/R64drd11 + ColE1 + pLG500

slot 3: GB15/R64drd11 + pLG540 + pLG500


1
2
3
chromosome
OC pLG500
CCC pLG500
OC pLG540
CCC pLG540

exhibits only very weak incompatibility with the ColE1-pLG500 composite plasmid.

It is clear from figure 8.3 that pLG500, which is efficiently complemented for relaxation by ColE1, is only rendered slightly relaxable by pLG540. This observation suggests that pLG540 either does not produce ColE1 relaxation proteins, or cannot provide them in trans, as efficiently as does ColE1. The results shown in figure 8.3 also suggest that the copy number of pLG500 is higher when pLG540 is present than when it is absent. However this observation is only tentative since no attempt to analyse it, even semi-quantitatively, has yet been made.

VI. Discussion

In this chapter I have examined the differences, and interactions, between the composite plasmids, pLG520 and pLG540, and their component replicons. pLG520 and pLG540 plasmids differ from ColK and ColE1 in several ways. Firstly, they display lowered levels of relaxation and mobilisation. I suggested earlier that this might be attributable to their possessing hybrid relaxation nicking sites, which because they were heterologous were less efficiently nicked by the ColE1 relaxation proteins. However, since pLG500 is rendered only slightly relaxable in the presence of pLG540, yet it is efficiently relaxed in the presence of ColE1, it seems more likely that pLG540 simply produces ColE1 relaxation proteins less efficiently than the parental ColE1 plasmid does. This is an interesting observation because it suggests that insertion of foreign DNA at the ColE1 relaxation nicking site may affect production of ColE1 relaxation proteins. It should in fact be possible to distinguish whether the composite plasmids are less susceptible to relaxation nicking by measuring the levels of relaxation

of pLG520 in the presence of ColE1, and of pLG540 in the presence of ColK.

pLG520 and pLG540 plasmids possess higher copy numbers, and, presumably as a result of this, encode higher colicin production and immunity levels, than their component Col plasmids. Models for copy number control by a plasmid coded repressor molecule predict that in a composite plasmid replication will normally proceed exclusively from the replication origin of the higher copy number component plasmid. For the composite plasmid pSC134, which consists of ColE1 and pSC101 joined together at their single EcoRI sites, this has indeed been found to be the case (Timmis, Cabello and Cohen, 1974; Cabello, Timmis and Cohen, 1976). Whilst I have not attempted to measure the copy number of pLG500, it may well be higher than that of ColE1 or ColK. If this is so then it is likely that the raised copy numbers of pLG520 and pLG540 (compared to ColK and ColE1) are a consequence of replication proceeding from the pLG500 replication origin in both of these composite plasmids.

Results presented here also demonstrate that pLG540 is incompatible with ColE1-Ap, but is apparently compatible with pLG500. The apparent compatibility of pLG500 and pLG540 is an unexpected finding, for in general it has been found that different derivatives of the same plasmid, and even of P15A, which has certain similarities to pLG500, exhibit incompatibility (see Chang and Cohen, 1978; Warren and Sherratt, 1978). Similarly it has been shown that the composite plasmid pSC134 is incompatible with both of its component replicons (Cabello, Timmis and Cohen, 1976). Rigorous experiments to determine whether pLG500 and pLG540 plasmids are absolutely compatible remain to be done, and I have not yet ruled out the possibility that the continued presence of pLG500 could be due to its production by the pLG540 plasmid, during vegetative growth.

Since pLG540 and ColE1 are incompatible this will influence the outcome of comobilisation experiments (no recipients containing both ColE1 and pLG540 are ever found following the comobilisation of ColE1 and pLG500). This means that the frequency with which composite plasmids are detected does not necessarily reflect the frequency with which they arise. The discrepancy between the level of detection of composite plasmids and the frequency with which they arise is also likely to be influenced by the probable copy number dependence of plasmid mobilisation. For example, it may be that during the comobilisation of ColE1 and pLG500 every donor cell produces at least one pLG540 plasmid, but since there are many more ColE1 and pLG500 molecules present, relatively few recipients actually receive the composite plasmids.

CHAPTER 9

GENERAL DISCUSSION

I. Conclusions from Studying the Mobilisation and Relaxation Properties of pLG500

The discovery of the cryptic miniplasmid, pLG500, in K216, and an analysis of its mobilisation and relaxation properties, raises several interesting points. In the past plasmids were classified simply as either conjugative or non-conjugative, and transfer (mobilisation) of the latter group was thought to occur passively. In recent years however, work with ColE1 has shown that (although an appropriate sex factor must also be present) this non-conjugative plasmid does play an active part in its own transfer, in that it codes gene products, and possesses a site, essential for mobilisation. In contrast, some other naturally occurring plasmids are known (for example, the P15A miniplasmid) that are non-mobilisable. Clearly therefore non-conjugative plasmids may be subdivided into different groups with regard to their transfer properties. pLG500, like P15A, is a non-mobilisable plasmid, yet it was originally found in a wild type strain which also carried ColK, and from which it was efficiently mobilised during R64*drd*11 mediated conjugation. This finding prompted experiments which confirmed that ColK complemented the mobilisation deficiency of pLG500. The fact that pLG500, which is itself a non-mobilisable plasmid, coexists in nature with ColK, which can complement it for mobilisation, is conceptually very important. It demonstrates that there may be a much greater diversity of transfer phenotypes amongst naturally occurring plasmids than was once imagined. Moreover, it strongly suggests that plasmids such as pLG500, although

they are strictly defined as non-mobilisable, may be efficiently transferred (at similar frequencies to the mobilisation proficient plasmid) in nature.

Previously (see Chapter 5) I mentioned that the naturally occurring P15A miniplasmid could be complemented for mobilisation by ColE1 (Chang and Cohen, 1978). The P15A miniplasmid came originally from a wild type strain of E.coli which, whilst it harboured two more plasmids (Ikeda, Inuzuka and Tomizawa, 1970), did not contain any other small plasmids (of a similar size to ColE1). Experiments to examine the trans effect of ColE1 on P15A would probably never have been performed if not for an earlier finding, that P15A shares substantial sequence homology with ColE1 (Goebel and Schrempf, 1972). Therefore the mobilisation properties of P15A were deduced by chance, and could not have been realised from a study of the wild type P15A-carrying isolate in the same way as was possible for pLG500. In turn, an analysis of the mobilisation properties of pLG500 raises the possibility that plasmid transfer hierarchies, consisting of a sex factor, a mobilisation proficient non-conjugative plasmid, and a Mob$^-$ plasmid (such as P15A or pLG500) may exist in nature. It would be interesting to examine the plasmid content and transfer properties of wild type colicin K and colicin E1 producing strains to see whether such combinations of plasmids exist. In view of this possibility it may be helpful to consider all of the natural isolates of E.coli that are studied as transconjugants of one or more matings, which may have received some, but not necessarily all, of the original parental plasmids.

An early objective of this thesis was to determine whether different plasmids could be found which could complement the mobility defect of Mob$^-$ ColE1 derivatives, and then to see whether they would also complement the relaxation deficiency. Whilst the experiments

using ColK and pML21 proved unsuccessful (in that complementation of the pML21 relaxation deficiency could not be demonstrated), an analysis of the mobilisation and relaxation properties of pLG500 inadvertently supplied the answer to the question posed for ColE1. The correlation between mobilisability and relaxability which was sought for pML21 was clearly demonstrated for pLG500 using ColK, ColE1, and ColE2. pLG500 seems to be a particularly good plasmid to use in such studies. Unlike pML21 none of its DNA is converted to the open circular form by SDS treatment, and therefore any trans effect on relaxability can be very easily detected using simple agarose gel electrophoresis techniques.

A striking feature of pLG500 is that it is mobilised at a significantly higher frequency than the Col plasmid which complements its mobility deficiency. Furthermore pLG500 appears to be a better substrate for relaxation nicking than the Col plasmid itself (more of it, than of the complementing plasmid, is converted to the open circular form by SDS treatment). Since virtually nothing is known about the frequency of relaxation nicking and transfer of individual plasmid molecules within a donor cell, it is impossible to tell whether the efficient transfer of pLG500 is a consequence of its very high copy number, or whether this miniplasmid is more efficiently nicked by ColE1-coded relaxation proteins than is ColE1 itself. Whatever the reasons for the high levels of relaxation, and very efficient mobilisation, of pLG500 in the presence of a complementing Col plasmid, these values are certainly much higher than those reported for several other Mob$^-$ plasmids. For example, Warren and Sherratt (1977) found that ColK-mediated complementation of the pML21 mobility defect resulted in a mobilisation frequency of 7.5% for the pML21 plasmid, and in comparable mating experiments I found that pML21 was mobilised

at a frequency of 10% (or 40% if glycerol was used as the carbon source). Similarly, when Warren et al. (1978) cloned part of the ColE1 genome containing its relaxation nicking site into an unrelated small plasmid, they found that this new derivative was mobilised at a frequency of 20%, and its relaxability was increased from 20% to 50% when ColE1 was present. In contrast, pLG500 is mobilised at a frequency of 100%, and virtually all of its DNA is rendered relaxable in the presence of ColE1 or ColK.

It was not possible to isolate ColK in the form of a relaxation complex using the method which had been devised for ColE1 (see Clewell and Helinski, 1969), and was later employed for several other plasmids. However, I was able to show that ColK, like ColE1, produced a large and easily detectable trans effect on the level of pLG500 relaxation. The close similarity of the effect produced by both ColK and ColE1 confirmed that the ColK plasmid did code relaxation protein equivalents. This was an important observation because it enabled the fact that ColK could complement the ColE1 mobility defect to be reconciled with the hypothesis that relaxation proteins have a central role in the process of plasmid mobilisation. A general implication from these findings is that even if a plasmid cannot be isolated in the form of a relaxation complex, it is still possible that it produces proteins which are functionally very similar to relaxation proteins.

It has been shown that the P15A miniplasmid shares sequence homology with ColE1, and that it can be complemented for mobilisation in the presence of this Col plasmid (Goebel and Schrempf, 1972; Chang and Cohen, 1978). Similarly, I have shown that pLG500 can be complemented for mobilisation and relaxation by ColK or ColE1, and this finding strongly suggests that pLG500 is closely related to ColK and ColE1 in

terms of sequence similarity. However pLG500, P15A, and ColK are all compatible with ColE1. This is an unexpected finding in view of the fact that different, genetically distinguishable, derivatives of the same plasmid (such as ColE1) can only be forced to coexist within the same cell if selection pressure for both plasmids is maintained. Furthermore, such plasmid incompatibility has been used to measure the degree of relatedness amongst natural isolates of conjugative plasmids (Datta and Hedges, 1971), and in general it has been found that plasmids belonging to the same compatibility group show sequence homology, whereas those in different compatibility groups show less, if any, sequence homology with one another (Guerry and Falkow, 1971; Grindley, Humphreys and Anderson, 1973; Ingram, 1973).

The list of small non-conjugative plasmids which share sequence homology and are compatible with ColE1 can probably be extended to include several more plasmids, known examples being CloDF13 and ColD. It is impossible to offer a completely satisfactory explanation for what seems to be a fundamental difference between the compatibility relationships amongst conjugative versus non-conjugative plasmids. However, in the case of pLG500, and by inference P15A as well, it is possible to rationalise the close relatedness and compatibility of these plasmids with ColK and ColE1 with the fact that they depend on the Col plasmids to supply gene products which are essential for their mobilisation. In short, it seems likely that the continued survival of these miniplasmids depends upon their ability to coexist with those closely related (mobilisation proficient) plasmids which can complement their mobilisation deficiency.

On a related theme, I have already discussed the fact that for several small non-conjugative plasmids the vegetative origin of replication and the relaxation nicking site are close together. In view of the

mobilisation and relaxation properties of pLG500 I suggested that the closeness of these sites might be evolutionarily favoured because it would enable all except the smallest derivatives of such plasmids to be rendered transmissible provided a related mobilisation proficient plasmid was also present. An alternative explanation which has been proposed to account for the closeness of the transfer origin and replication origin of ColE1 and other small plasmids is that relaxation nicking at the transfer origin is necessary in order to divert replication from the Cairns to the Rolling Circle mode. Neither this suggestion, nor the one I proposed, seem so attractive when the structure of conjugative plasmids is also taken into consideration. For some conjugative plasmids, such as F, the transfer origin and the replication origin are known to be well separated. Recently evidence has been obtained that the relaxation nicking site and the transfer origin of the conjugative plasmid RK2 are at least roughly coincident (Guiney and Helinski, 1979), and these sites are known to be separated from the vegetative replication origin by about 20 kilobases. Thus although it seems likely that relaxation complexes of both conjugative and non-conjugative plasmids are involved in transfer, the location of the relaxation nicking site (with respect to the origin of vegetative replication) is quite different for these different types of plasmids.

II. Implications of the RecA Independent Recombination Phenomenon Involving pLG500 and ColK or ColE1

The second main theme of the experimental work reported in this thesis is the discovery and characterisation of a novel type of recA independent recombination, which was found to occur between pLG500 and ColK or ColE1. As I discussed previously, this illegitimate recombination phenomenon is intimately associated with the plasmid mobilisation process, and, based on the currently favoured hypothesis that ColE1 transfer entails single-stranded DNA transfer from, and strand recircularisation at, relaxation nicking sites, cointegration can be explained as a direct consequence of ColK or ColE1-coded relaxation proteins complementing the pLG500 mobilisation deficiency. It is not clear whether such recombination occurs specifically during plasmid transfer, or whether it occurs prior to transfer (in vegetatively growing cells) as a result of aberrant relaxation nicking and abortive mobilisation events. However, since the structure of these recombinants is completely compatible with their being the hybrid products of comobilising different plasmids using functionally identical mobilisation proteins, an analysis of their properties may provide valuable information about the plasmid mobilisation process. Since all previous studies of plasmid mobilisation have been confined to an examination of the events which occur prior to transfer, a study of such recombinants, reflecting events which occur after relaxation nicking in the donor, may provide a useful new perspective on this process.

When Warren and Sherratt (1977) demonstrated that the presence of the plasmid ColK stimulated transfer of the Mob$^-$ ColE1 derivative, pML21, they attempted to show that this occurred as a consequence of the provision of ColK-coded gene products, and that it was not simply due to recombination of pML21 with the mobilisation proficient ColK plasmid

occurring in the donor. This was an important distinction to make because ColK was known to share significant sequence homology with ColE1. Therefore matings were performed using a recA donor strain, and subsequent experiments were done to confirm that kanamycin resistant recipients retained their Mob$^-$ phenotype.

Receipt of the original parental plasmids as independent replicons has remained an important criterion in demonstrating complementation of the mobility defect of Mob$^-$ plasmid derivatives. However when ColK and pLG500, or ColE1 and pLG500, are comobilised some recombination can be detected following transfer from a recA donor to a recA recipient, and an analysis of the properties of the cointegrate plasmids which are formed suggests that such recombination occurs as a direct consequence of the Col plasmid complementing the mobilisation deficiency of pLG500. Indeed, according to the model of single-stranded DNA transfer during plasmid mobilisation, such linkage might be expected to occur between any plasmids which code functionally identical mobilisation proteins, or between any pair of plasmids where one complements the mobility defect of the other.

Although it is not known whether recombination between pLG500 and ColK or ColE1 occurs specifically during transfer, circumstantial evidence suggests that this is very likely. Whilst the composite plasmids, pLG520 and pLG540, are detected at quite low frequencies following comobilisation, this does not necessarily mean that the ligation event which generates them is also rare. One factor which I have already mentioned that would affect the frequency with which composite plasmids would be detected is incompatibility between these novel plasmids and their component Col plasmids. Similarly, it may be that certain combinations of plasmids yield inviable cointegrates. Although I found composite plasmids consisting of pLG500 linked to

either Col plasmid, no dimers of pLG500, ColK, or ColE1 were ever found. It is possible that the latter types of plasmid cointegrates are formed in matings between recA donors and recipients, yet they are not detected because they fail to replicate properly, and are subsequently diluted out of the population. Moreover, if, as seems likely, the composite plasmids are formed by ligation of the 3' and 5' ends of different plasmid strands at their relaxation nicking sites, it is possible that many of the cointegrates which are formed are later resolved (once again yielding the component replicons) by the nickase enzyme of the relaxation complex.

In conclusion, it seems likely that end to end ligation of transferring plasmid molecules is a normal feature of the plasmid mobilisation process. Whilst complementation of the mobility defect of a plasmid seems to depend upon the provision of gene products, and not on recombination, the actual process of complementation may result in the formation of viable composite plasmids. Finally, the possibility that the type of recombination I have described actually plays a role in stimulating the transfer of Mob^- plasmid derivatives cannot be ignored. It is possible that such recombination accounts for the transfer of pLG500, but that in most instances the cointegrates which effect the transfer of pLG500 are resolved by relaxation nicking, and only rare recombinants, which by chance escape the action of the nickase, are found.

In 1978 Warren et al. proposed a model for ColE1 transfer which was based on an analogy between the activity of the ØX174 cisA protein and the presumptive nickase-ligase enzyme of the ColE1 relaxation complex. Extensive studies of ØX174 in vitro replication (for example, see Eisenberg et al., 1977) have shown that this phage encodes production of a multifunctional enzyme - the cisA protein - which

binds to and nicks ØX174 phage DNA at its replication origin. Endonucleolytic nicking of the ØX174 duplex is followed by rolling circle replication, during which the cisA protein (which is covalently attached to the 5' end of the linear strand) remains locked in the replication fork, and participates both in strand unwinding and replication. Following a round of replication the cisA protein nicks again at the regenerated replication origin, and ligates the ends of the displaced single strand to form a viral circle.

Whilst the nickase-ligase activity of the cisA protein may be very similar to that of the 60K ColE1 relaxation protein, the analogy between ØX174 replication and ColE1 transfer replication is unlikely to be helpful in deducing details of the ColE1 mobilisation process. The important difference between these two processes is that, unlike ØX174 replication, rolling circle replication of ColE1 occurs during transfer, and may not therefore only involve events within a single cell. In turn, it seems inevitable that these two processes, even if they reflect some distant ancestral relationship between ColE1 and ØX174, are likely to have become substantially different, because of these different evolutionary constraints. It has anyhow been possible for some time now to propose various models for ColE1 mobilisation, based on a comparison with the known features of conjugal DNA transfer, and all incorporating the basic premise of single-stranded DNA transfer from, and strand recircularisation at, the relaxation nicking site. In the past, before ColE1 was known to play an active part in its own transfer, the properties of its relaxation complex were even used to explain how single-stranded DNA transfer might be accomplished for the sex factor F (see Kline and Helinski, 1971). The importance of the experiments I have reported, describing the phenomenon of recA independent recombination between pLG500 and ColK or ColE1, is that

the properties of the resulting pLG520 and pLG540 cointegrate plasmids confirm the predictions of all of the above such models for the mobilisation of ColE1 and related plasmids. Finally, I should emphasise that it is not possible to extrapolate any details of the ColE1 mobilisation process (such as timing of complementary strand synthesis and recircularisation of transferring strands) either from my findings or from any previous work, and very probably these aspects will not be resolved until this process can be studied at the molecular level, either *in vivo* or *in vitro*.

I have already shown that the ligation of different plasmid strands at their relaxation nicking sites is a predicted outcome of complementation of the mobility defect of one plasmid by the other. There is no reason to suppose that this phenomenon is exclusive to pLG500. The generation of similar composite plasmids could therefore provide a general strategy for the identification of relaxation nicking sites and transfer origins, and it might be applicable not only to non-conjugative plasmids, but also to small derivatives of conjugative plasmids. Indeed, to some extent, I have used this approach in the analysis of pLG520 and pLG540 plasmids. For example, in the case of ColK, where no relaxation complex has been isolated, and the relaxation nicking site has not been identified, it was possible to show that the site of *recA* independent recombination with pLG500 was within the same restriction fragment as the ColK *bom* site. By further restriction analysis it should be possible to localise the site for recombination to within a very small restriction fragment (for example, of the order of 50-100 base pairs), and then to compare the sequence of this piece of DNA to that of the ColE1 relaxation nicking site.

One fascinating aspect of the recombination phenomenon I have described is that it was found to occur between two plasmids, ColK and pLG500, which coexist in nature. Just as it is possible that ColK and pLG500 are comobilised in the wild, in turn it is likely that they sometimes undergo site-specific, recA independent, recombination to form pLG520 plasmids. pLG520 encodes much higher levels of colicin K production than the ColK plasmid. Whilst colicinogeny has been difficult to study in vivo, it does seem to provide a selective advantage (for a brief review see Hardy, 1975), even despite the fact that some colicins are known to be inactivated by proteases present in the intestine. Whether pLG520-carrying cells are therefore at a selective advantage over ColK-carrying cells remains open to speculation. A more important implication of this recombination phenomenon is that it may represent a novel way of reassorting plasmid molecules in vivo. Until the discovery of illegitimate recombination events, such as transposition, plasmid evolution was assumed to progress mainly by homologous recombination between closely related sequences. It is now clear that the acquisition of new genetic material also occurs by illegitimate recombination between plasmid DNA sequences with little or no ancestral relationship. Whilst the mechanism of recombination I have described is a specialised process, it may still represent yet another way of reassorting plasmid molecules by protein-DNA, rather than DNA-DNA, interactions.

III. General Conclusions about the Role of Relaxation Complexes and the Mechanism of Plasmid Mobilisation

The aim of this project was to ascribe a physiological role to plasmid relaxation complexes, initially at least by investigating the properties of the ColE1 complex. The work I have presented strongly supports the hypothesis that ColE1 relaxation proteins have a central role in the plasmid mobilisation process, firstly by demonstrating a correlation between the mobilisability and relaxability of wild type ColE1, and also by showing that ColE1 complements both the mobilisation and relaxation deficiency of a naturally occurring miniplasmid, pLG500. My results also demonstrate a central role for relaxation proteins in the transfer of two more plasmids, ColK and pLG500, which are closely related to ColE1.

Whether all plasmid relaxation complexes will eventually be shown to be involved in plasmid transfer remains to be seen. One anomaly which comes to mind is that a deletion mutant of F which lacks the transfer origin is known that can still be isolated as a relaxation complex (Guyer and Clark, 1976). Moreover, Novick (1976) has found that several small plasmids of S.aureus can be isolated in the form of relaxation complexes, yet no conjugation system has ever been demonstrated for this organism. The enzymic activity of the ColE1 relaxation complex has been compared to that of the ØX174 cisA protein, and the replication processes which these enzymes participate in are similar except one involves transfer replication and the other vegetative replication. The analogy has also been drawn between plasmid relaxation complexes and other protein-DNA complexes, such as the SV40 complex, the proteins of which may be involved in vegetative replication or transcription. Therefore for those plasmids where a correlation between relaxation and mobilisation cannot be demonstrated

the possibility remains that the relaxation complex does not have a role in transfer, but its presence reflects a distant ancestral relationship with these other extrachromosomal elements and it might either be redundant or have evolved some different physiological function.

At least for ColE1 and related plasmids it is clear that relaxation proteins have a central role in plasmid mobilisation. Whilst other authors have obtained indirect evidence suggesting that the ColE1 relaxation nicking site is also its transfer origin, the experiments I have reported demonstrate that a recombination, or nickase, event occurs *in vivo*, either at or very near the ColE1 relaxation nicking site. The products of such recombination very probably reflect those events which occur during plasmid mobilisation, after relaxation nicking has occurred, and an analysis of such composite plasmids can therefore provide a new perspective on the topography of the plasmid mobilisation process.

It may not be possible to elucidate the molecular details of the ColE1 mobilisation process for some time yet. However, work from other laboratories has revealed some unsuspected, novel, features of the mobilisation process, which are amenable to genetic and biochemical analysis. For example, it has recently become clear that the 60K ColE1 relaxation protein is too large to be coded by the ColE1 mobility region unless overlapping genes are involved. Therefore it will be interesting to see whether this protein is plasmid-coded, or whether it is a product of the host genome. Moreover, it has been shown that mobilisation of wild type ColE1 by some sex factors (such as RP4) requires extra ColE1-coded proteins, in addition to those coded in the mobility region which is essential for F or R64*drd*11 mediated transfer. Since RP4-promoted mobilisation of ColE1 and pLG500 resulted

in complementation of the pLG500 mobilisation deficiency, and in the formation of a composite plasmid which was identical to pLG540, this indirectly suggests that the conditional mobility proteins do not participate in an alternative mechanism of transfer, but rather that they are involved in creating the correct conditions for mobilisation to occur. It is possible that the sex factors which promote ColE1 mobilisation act not only in initiating this process, but also in protecting plasmid strands during transfer, or in strand recircularisation. These complex interactions between sex factors and non-conjugative plasmids, which are necessary for mobilisation to occur, will be a fascinating area for future investigations.

CHAPTER 10

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