BACTERIAL PLASMIDS: AUTONOMOUS REPLICATION AND VEHICLES FOR GENE CLONING

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INTRODUCTION

Plasmids are circular duplex DNA molecules that are stably maintained in an extrachromosomal state in a bacterial cell. The duplication of these elements is being studied in a number of laboratories at both the in vitro and in vivo level. Interest in plasmid elements stems from the diverse set of important phenotypic properties of the bacterial cell that they determine, as well as their use as model systems for analyzing the regulation of duplication of circular DNA elements in general. Considerable interest also has been generated in plasmid elements as a result of their wide-spread use as molecular vehicles for the cloning of foreign DNA in bacteria.

This presentation will be concerned largely with our own studies on the replication properties of plasmids. Recombinant DNA techniques have provided a powerful new approach toward understanding the control of replication of plasmid molecules. Conversely, the use of these techniques in the analysis of plasmid structure and replication has resulted in the construction in vitro of new plasmid elements that are more effective or biologically containable in their employment as molecular vehicles for gene cloning. The properties of several of these plasmid derivatives potentially useful as cloning vehicles will be described.

Plasmid elements are found in most species of either Gram-positive or Gram-negative bacteria. These elements specify a wide variety of phenotypic properties that are not required for normal cell growth or division, but often provide the cell with a substantial advantage or a vital property in a particular environment. For example, plasmid genes are responsible for most instances of resistance to antibiotics displayed by pathogenic and nonpathogenic bacteria. A plasmid can be classified into one of two major groups, conjugative and nonconjugative, depending upon whether or not it carries a functional set of genes that promote bacterial conjugation. In addition, as shown in Table 1, a plasmid element can be given a specific designation on the basis of identifiable products specified by the plasmid. For example, if a plasmid possesses genes involved in the production of colicins (extracellular, antibiotically active proteins produced in *Escherichia coli*), it is designated a colicinogenic, or Col plasmid. If it carries a gene determining resistance to an antibiotic, then the plasmid is designated an antibiotic resistance or R plasmid.

The overall architecture of the two major types of plasmids is shown in Figure 1. To date, all plasmids isolated from either Gram-positive or Gram-negative bacteria are found in the form of covalently closed, duplex circular DNA molecules. In the case of several plasmids examined,[1-9] the genes responsible for their autonomous replication have been observed to be clustered within a relatively small segment of the molecule that includes the region of the origin of vegetative replication. Similarly, the genes responsible for the conjugative or transfer properties of a plasmid are clustered.[10,11] Although the essential genes for the maintenance of the autonomous state of plasmids usually are clustered, this is not the case for every plasmid element. As shown below, the plasmid RK2 has its essential genes for replication distributed over a relatively large portion of the circular DNA molecule.[12]

TABLE 1

Conjugative and Nonconjugative Plasmids

Plasmid class	Genetic regions of plasmid element		Plasmid type
	Common	Specific	
Nonconjugative	Autonomous replication	Colicin production	Colicinogenic (Col) plasmids
		Antibiotic resistance	R plasmids
Conjugative (sex factors)	Autonomous replication and transfer (tra)	Colicin production	Colicinogenic (Col) plasmids
		Antibiotic resistance	R plasmids
		Enterotoxin production	Ent plasmids

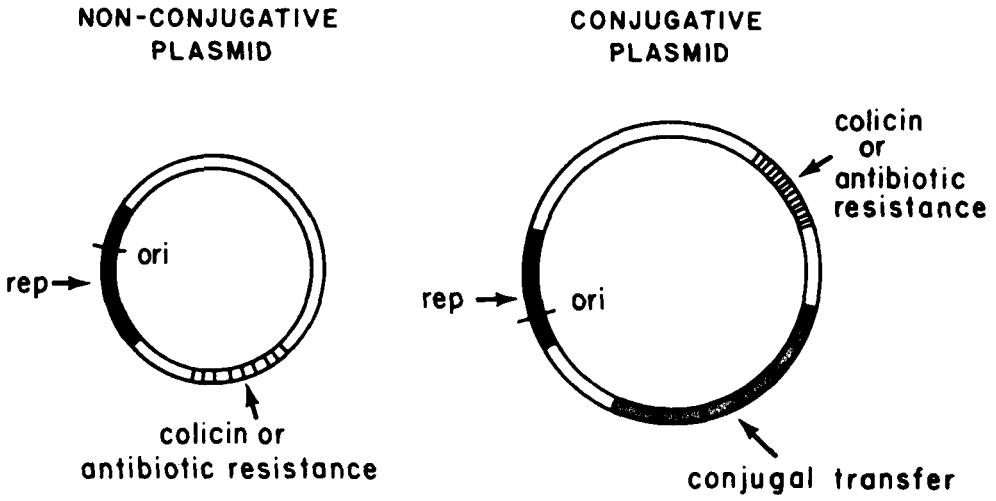

FIGURE 1. Structure of nonconjugative and conjugative plasmids. The cluster of genes essential for replication of the plasmid is indicated (*rep*) as well as the origin (*ori*) of replication. In addition to colicin production and antibiotic resistance, a variety of other bacterial traits are specified by genes carried on a plasmid element.

ORIGIN AND DIRECTIONALITY OF PLASMID REPLICATION

The origin and direction of replication of plasmid molecules has been determined by an electron microscopy analysis of replicating circular DNA forms of plasmids isolated from bacterial cells growing in the logarithmic state. The parental strands of a replicating plasmid molecule remain covalently closed and, therefore, replicating molecules are generally broadly distributed between the densities of supercoiled and open circular DNA in a cesium chloride-ethidium bromide density gradient. As shown in Figure 2, these replicating molecules appear on electron microscopy as partially supercoiled, partially open structures. One can cleave these molecules with a specific restriction endonuclease that cleaves the plasmid at a unique site. Electron microscopy analysis of these cleaved molecules allows a comparison between the positions of the replication forks and the unique restriction enzyme site. Replicating molecules of the plasmid ColE1, cleaved with the restriction enzyme EcoRI, are shown in Figure 3.

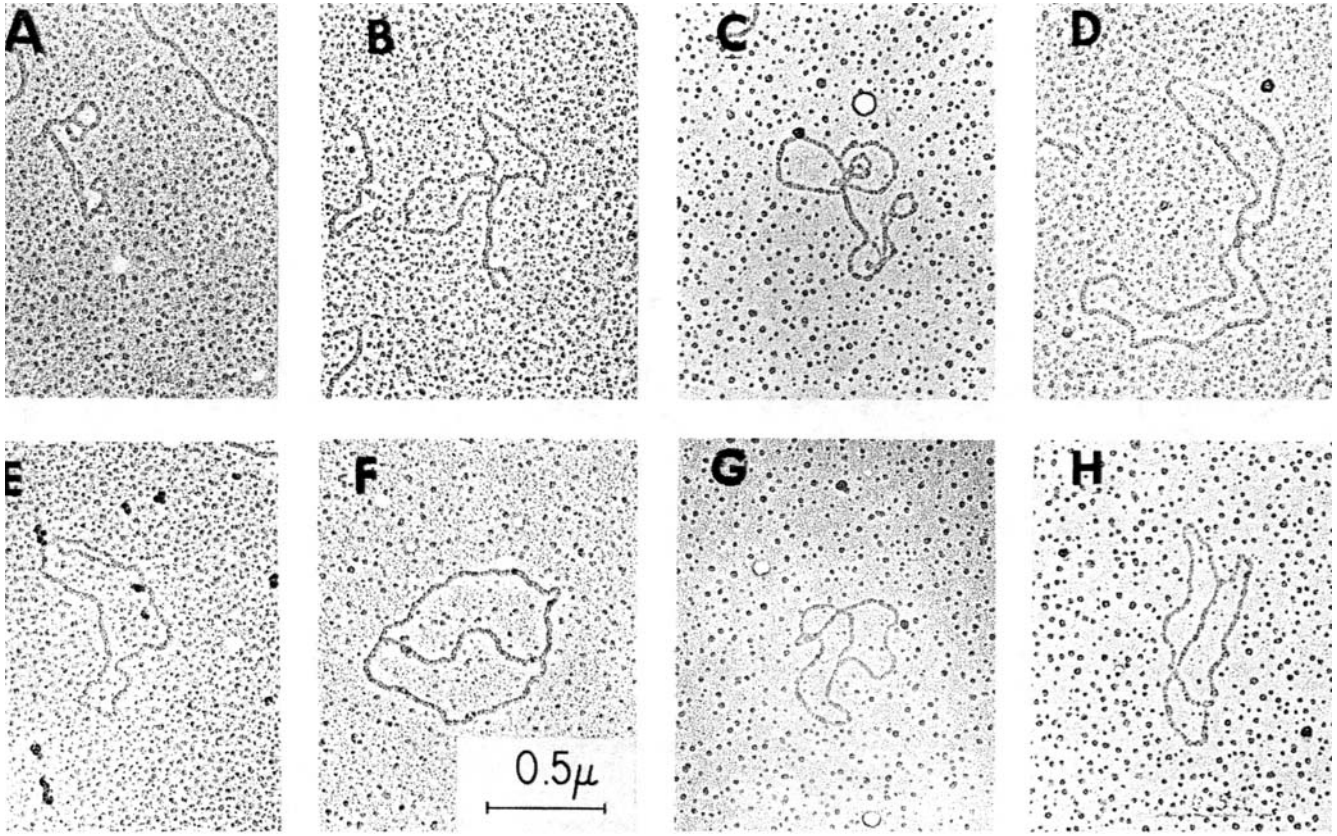

FIGURE 2. Replicating forms of ColE1 DNA. Electron micrographs of selected molecules after no prior treatment (A, B, C, and D) or treatment to introduce at least one nick in a parental strand (E, F, G, and H). (Reprinted with permission from Katz, L., Williams, P. H., Sato, S., Leavitt, R. W., and Helinski, D. R., *Biochemistry*, 16, 1677, 1977. Copyright by the American Chemical Society.)

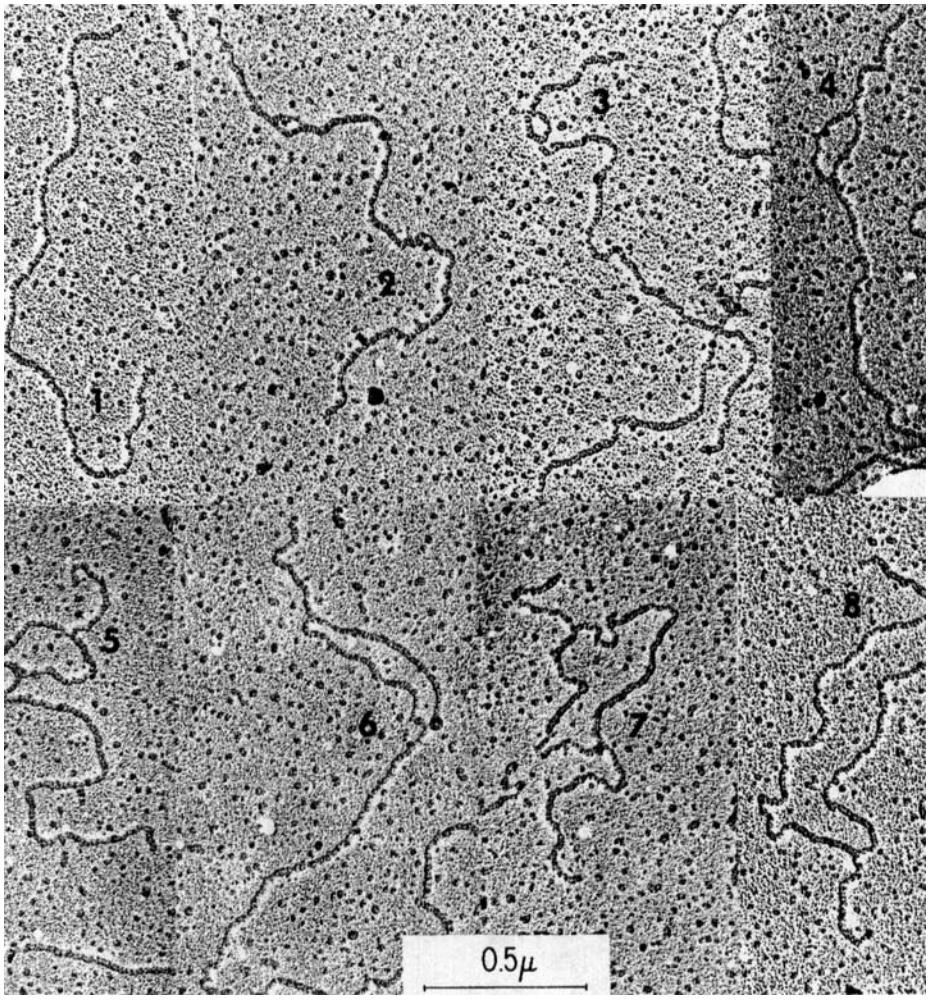

FIGURE 3. Replicating ColE1 DNA molecules treated with the restriction endonuclease EcoRI. Electron micrographs of selected molecules are arranged in order of increasing extent of replication. Panel 1 represents an unreplicated linear molecule. (Taken from Lovett, M. A., Katz, L., and Helinski, D. R., *Nature (London)*, 251, 337, 1974. With permission.)

Plotting the positions of the replication forks in molecules replicated to various extents demonstrates unidirectional replication of plasmid ColE1 from an origin that is approximately 18% of unit length from one EcoRI end of the molecule[13] (Figure 4). A similar analysis of replication of plasmids R6K, mini-F (a low molecular weight plasmid derivative of the Flac plasmid), and RK2 has shown a bidirectional mode of replication of R6K[16] and mini-F[17] and unidirectional replication for plasmid RK2.[18]

PLASMID INCOMPATIBILITY GROUPS

A bacterial cell can maintain more than one type of plasmid molecule. However, certain pairs of plasmids cannot be stably maintained by a cell. On the basis of the inability of one particular plasmid to be stably maintained in the same cells with another, plasmids have been combined into a number of different incompatibility groups. Over 30 incompatibility groups have been described for plasmid elements found in Gram-negative bacteria.[19] In general, any member of the same incompatibility group cannot stably coexist in a bacterial cell with any other member of that group, but it

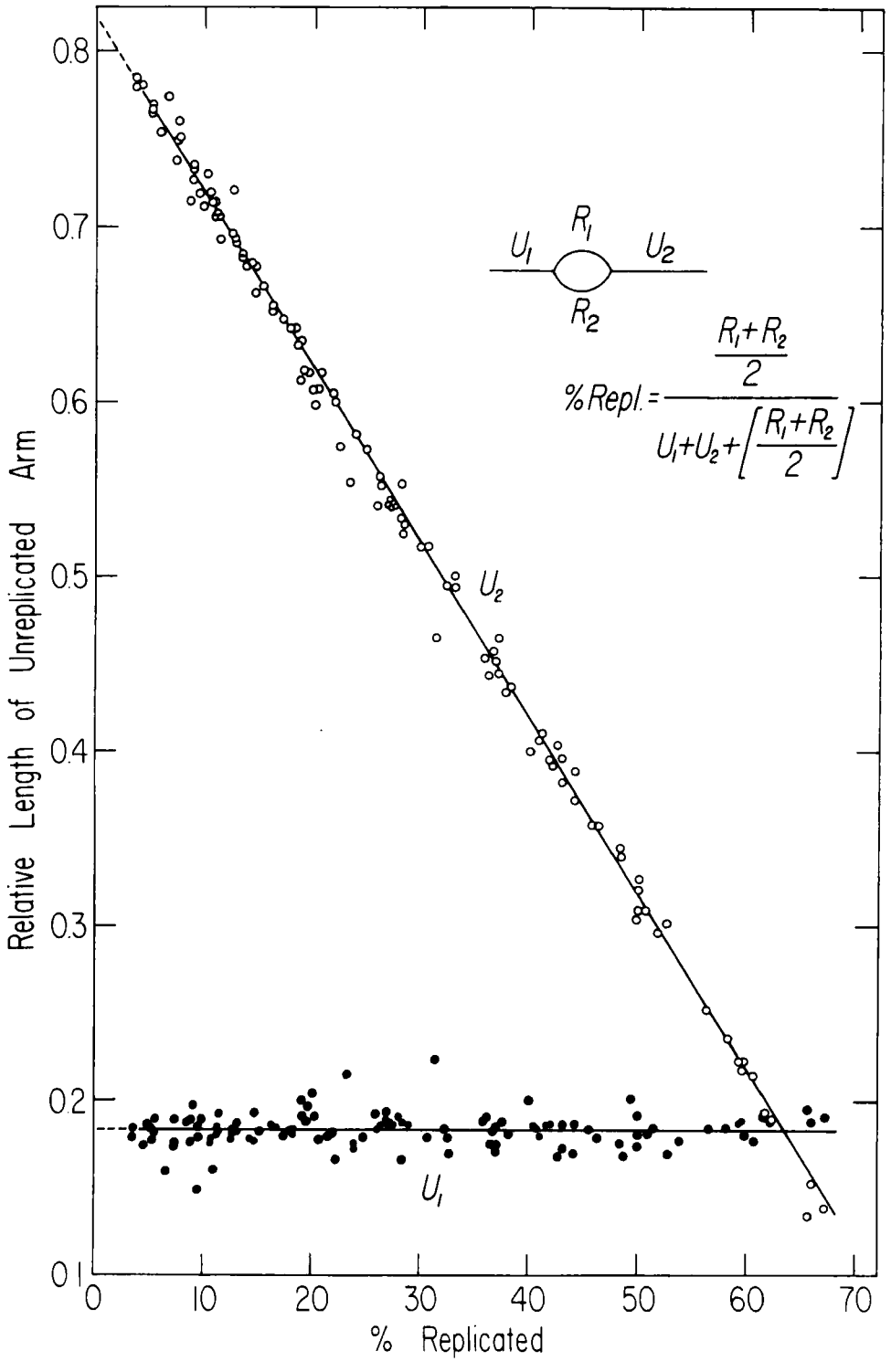

FIGURE 4. Determination of directionality and position of origin of replication. The lengths of the two unreplicated segments of replicating ColE1 DNA molecules were determined after cleavage with EcoRI. This method was used earlier to determine the origin of replication of SV40 DNA.[15] (Taken from Lovett, M. A., Katz, L., and Helinski, D. R., *Nature (London)*, 251, 337, 1974. With permission.)

can stably coexist with any member of a different incompatibility group. One particular incompatibility group, the P-1 group, is of special interest in that members of this group exhibit broad host range properties, i.e., they are capable of being stably maintained in a wide variety of Gram-negative bacteria.[20-22] This is in contrast to the relatively narrow host range of most other naturally occurring plasmids identified in *E. coli* and other bacterial species. The broad host range properties of P-1 group plasmids make them particularly suitable for development as molecular vehicles for gene cloning in bacteria distantly related to *E. coli*, as for example, the agriculturally important genera *Rhizobium* and *Agrobacterium*.[23]

STRINGENT AND RELAXED PLASMIDS

It has been generally observed that the low molecular weight plasmids (e.g., ColE1 and other plasmids of this type shown in Table 2) are maintained as many copies per chromosome. These plasmids, with the exception of the R6K plasmid shown in Table 2, are generally nonconjugative in that they do not possess a functional set of genes required for conjugal mating and the intercellular transfer of DNA. In contrast, the relatively high molecular weight plasmids are of the conjugative type and are maintained as a limited number of copies per cell (Table 2). An unusual feature of certain members of the multicopy group of plasmids, as initially demonstrated for ColE1,[25,34,35] is the ability of these plasmids to continue to replicate in the presence of the protein synthesis inhibitor chloramphenicol. In the presence of this inhibitor, chromosomal DNA synthesis ceases after a short period of time, but plasmid replication continues and results in the accumulation of a large number of copies of the plasmid per cell. This property of certain multicopy plasmids has been taken advantage of in their use as cloning vehicles for recombinant DNA research, since the plasmid element or a foreign DNA insert in the plasmid can be obtained in very high yield after "amplification" of the molecule in the presence of chloramphenicol.[36]

USE OF PLASMIDS AS CLONING VEHICLES

Since the initial demonstration of the effectiveness of a plasmid for the cloning of genes in *E. coli*, a variety of plasmid elements have been developed as cloning vehicles in both *E. coli* (see Reference 37) and the Gram-positive bacterium *Bacillus subtilis*.[38-40] These cloning vehicles have in common many of the following properties that are advantageous for the cloning of foreign DNA from the perspective of efficacy and biological containment of the recombinant molecule: (1) stable maintenance in the host bacterial cell; (2) non-self-transmissibility; (3) low molecular weight and presence as multiple copies per cell; (4) ease of joining with and replicating foreign DNA of a broad size range; (5) amplification upon incubation of cells harboring the hybrid plasmid in the presence of chloramphenicol; and (6) the presence of one or more genes useful as selective markers for the transformation of a bacterial cell.

Plasmid ColE1, its derivatives pCR1 and RSF2124, and the ColE1-like plasmids pMB9, pBR313, and pBR322 have been frequently used as molecular vehicles in gene cloning experiments, since these plasmids normally are maintained in the *E. coli* cell in the multicopy state and are amplified in the presence of the protein synthesis inhibitor chloramphenicol (see Reference 37). As shown in Figure 5, it is possible to amplify the level of a covalently closed circular DNA form of a hybrid plasmid of ColE1 to the extent of approximately 40 to 50% of the total cellular DNA (equivalent to as many as 2000 copies of the hybrid plasmid per cell, depending upon the size of the foreign DNA insert).[36] Using this technique, *E. coli* cells grown in liquid culture can provide a very abundant source of a specific segment of DNA or a specific gene inserted into the plasmid vehicle.

TABLE 2

Stringent and Relaxed Naturally Occurring Plasmids

Plasmid	Size (daltons)	Self-transmissibility	Number copies per chromosome	Amplification + CAM*	Other characteristics	Ref.
ColE1	4.2×10^6	−	10—15	+	Colicin E1	24,25
RSF1030	5.6×10^6	−	20—40	+	Ap^R	26
CloDF13	6.0×10^6	−	10	+	Cloacin DF13	27
R6K	25×10^6	+	13—38	−	Ap^{R}Sm^R	28
F_1	62×10^6	+	1—2	−	—	11,29
R1	65×10^6	+	1—3	−	Ap^{R}Cm^{R}Su^{R}Sm^{R}Kn^R	30,31
RK2	38×10^6	+	5	−	Ap^{R}Kn^{R}Tc^R	32
EntP307	65×10^6	+	1—3	−	Enterotoxin	33

* Refers to ability of plasmid to continue to replicate in the presence of chloramphenicol (CAM).

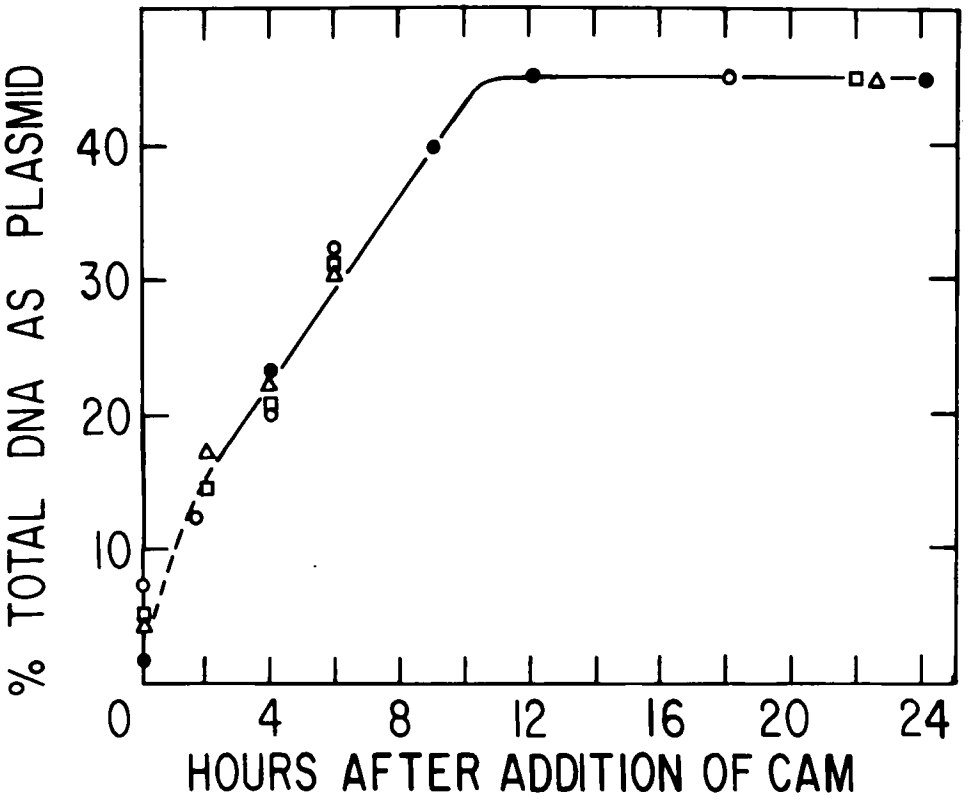

FIGURE 5. Accumulation of ColE1 and ColE1 hybrid plasmids in cells incubated in the presence of chloramphenicol (CAM). *Escherichia coli* cells carrying plasmids ColE (o—o), a ColE1 hybrid carrying a segment of λ bacteriophage plus *E. coli* tryptophan operon DNA(□—□ and ●—●), and a ColE1 hybrid carrying the kanamycin resistance fragment described in Figure 6 (△—△) were grown and samples removed at 0, 2, 4, 6, and 22 hr after the addition of CAM. (Taken from Hershfield, V., Boyer, H. W., Yanofsky, C., Lovett, M. A., and Helinski, D. R., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 3455, 1974. With permission.)

CONSTRUCTION OF LOW MOLECULAR WEIGHT DERIVATIVES OF NATURALLY OCCURRING PLASMIDS

It is clear that plasmid elements have greatly facilitated the use of recombinant DNA techniques to establish foreign DNA in a bacterial cell. In turn, recombinant DNA technology has been used to great advantage to reduce the size of a naturally occurring plasmid molecule for the purpose of obtaining low molecular weight derivatives that are more readily manipulated in experiments directed towards the analysis of genetic and biochemical factors regulating the maintenance of a plasmid element in the autonomous state. In addition, recombinant DNA techniques have been used with considerable success for the purpose of constructing plasmid derivatives that are more effective as cloning vehicles. The success with the recombinant DNA approach in reducing the size of a plasmid element stems mainly from the finding for several different plasmids that the genes or genetic regions required for maintenance of the plasmid in an automomous state are clustered in a relatively small region of the plasmid molecule. Consequently, utilizing the approach described in Figure 6, a mini-F derivative (9-kilobase [kb] segment) was obtained from the plasmid Flac (approximately 140 kb).[1,2] Basically, this method consists of joining a non-self-replicating restriction enzyme fragment of DNA which contains an antibiotic resistance gene to a total digest of the parental plasmid prepared with the same restriction enzyme and selecting the fragment of DNA carrying the replication region by transformation of *E. coli* cells for the antibiotic resistance. In the experiment described in Figure 6, the segment of DNA specifying resistance to the antibiotic kanamycin was obtained by EcoRI digestion of the antibiotic resistance plasmid pSC105. In the case of the Flac plasmid, the essential genes for maintenance of this plasmid in the autonomous state were found to be clustered on a 6×10^6 dalton fragment.[1,2] This mini-F derivative of Flac is under stringent replication control (one to two copies per chromosome) and exhibits the incompatibility properties of the parental plasmid.

As indicated in Figure 7, the origin of replication of a kanamycin-resistant derivative of mini-F occurs within the mini-F fragment at position 42.6 kg (kilobase units correspond to the physical map of the F1 plasmid).[17] Replication was found to be predominantly bidirectional from this origin by an electron microscopy analysis similar to that described above for the ColE1 plasmid. Also as shown in Figure 7, large portions of the mini-F plasmid have been removed in vitro with several different restriction endonucleases to define more precisely the regions essential for maintenance of this plasmid in the autonomous state. One surprising finding from this approach was that the plasmid will replicate after removal of the 2.4 kb BamHI fragment containing the mini-F origin at 42.6 kb.[42] In this situation, the cell utilizes a second site (origin) of mini-F for the initiation of replication. This secondary origin, located at 44.4 kb, is used to maintain the plasmid in the absence of the primary origin at 42.6 kb. Thus, as found earlier for the plasmid R6K,[43] a plasmid can possess more than one functional origin of replication. In the case of the mini-F plasmid, utilization of the origin at 42.6 kb is preferred when both origins are present.

Palchaudhuri and Maas[44] have mapped a region between 46.4 and 48.6 kb that appears to be responsible for the incompatibility properties of the F plasmid. The physical map of mini-F shown in Figure 7 indicates that the origin of replication at the 42.6 kb position is itself not responsible for incompatibility. Further analysis of the various deletions of the mini-F plasmid constructed in vitro have indicated that derivatives of the size of 3 kb can be obtained that are maintained in the autonomous state with the same copy number and incompatibility properties of the parent F plasmid. These studies clearly indicate that a region that is 1/30 the size of the F plasmid (molecular weight

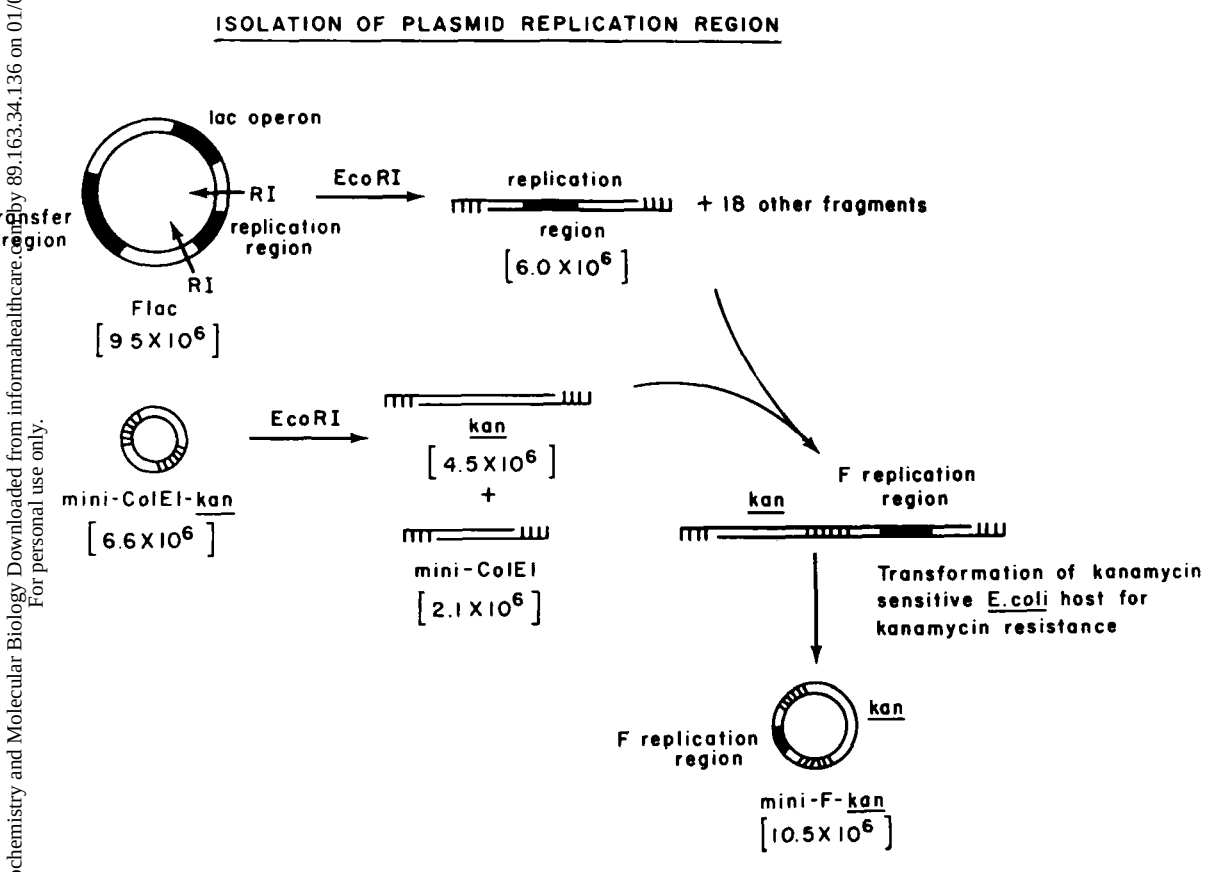

FIGURE 6. The method employed is as described in Lovett and Helinski.[2] A similar procedure has been described by Timmis et al.[1] Values in brackets refer to the molecular weight of the plasmid or DNA fragment.

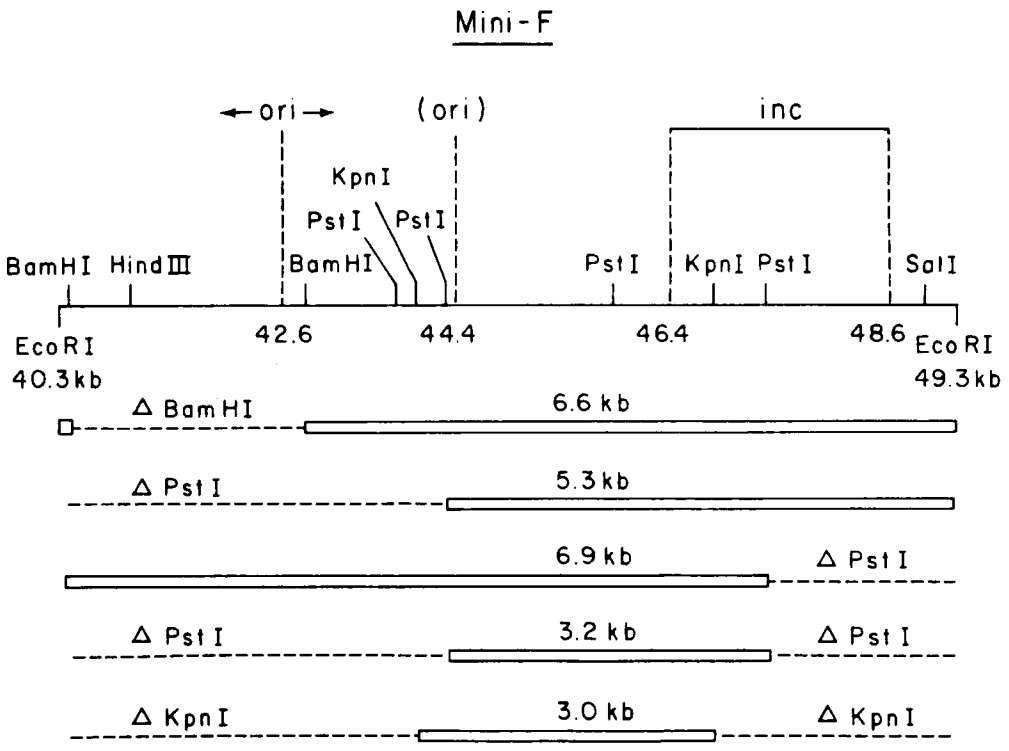

FIGURE 7. Physical and genetic map of plasmid mini-F. The cleavage sites for several restriction endonucleases are shown relative to the kilobase coordinates on the physical map of F.[11] Ori refers to the origin of replication and inc defines the incompatibility region. The extent of various deletions of mini-F is shown by the dashed lines. (Taken from Figurski, D., Kolter, R., Meyer, R., Kahn, M., Eichenlaub, R., and Helinski, D. R., in *Microbiology 1978*, Schlessenger, D., Ed., American Society for Microbiology, Washington, D.C., 1978, 105.

of 62×10^6) carries the essential information for the autonomous replication, stringent regulation of copy number, and incompatibility properties of the F plasmid. The coding capacity of this region, assuming the absence of overlapping genes, is approximately 100,000 daltons of protein.

COLE1 DERIVATIVES

The functional region of plasmid ColE1 with respect to autonomous replication has been identified relative to the cleavage sites of EcoRI, Pst 1, and the Hae II restriction endonucleases, as shown in Figure 8.[45,47] ColE1 replicates unidirectionally from an origin of replication most likely located in the Hae II-E fragment.[46] The first characterized derivative of ColE1, designated mini-ColE1, was obtained spontaneously in cells harboring an in vitro constructed ColE1 hybrid plasmid carrying portions of the λ bacteriophage and the tryptophan operon of *E. coli*.[3] The portion of ColE1 remaining in mini-ColE1 is indicated in Figure 8. The mini-ColE1 plasmid displays properties identical to ColE1 with respect to its dependence on DNA polymerase I, replication in the presence of chloramphenicol, and ColE1 immunity. This ColE1 derivative does not, however, specify the production of colicin E1, since as shown in Figure 8, it lacks the structural gene for this antibiotically active protein. The mini-ColE1 plasmid also cannot be isolated in the form of a relaxation complex.[3] Additional regions of ColE1 not essential for replication were determined by the production of deletions in vitro in

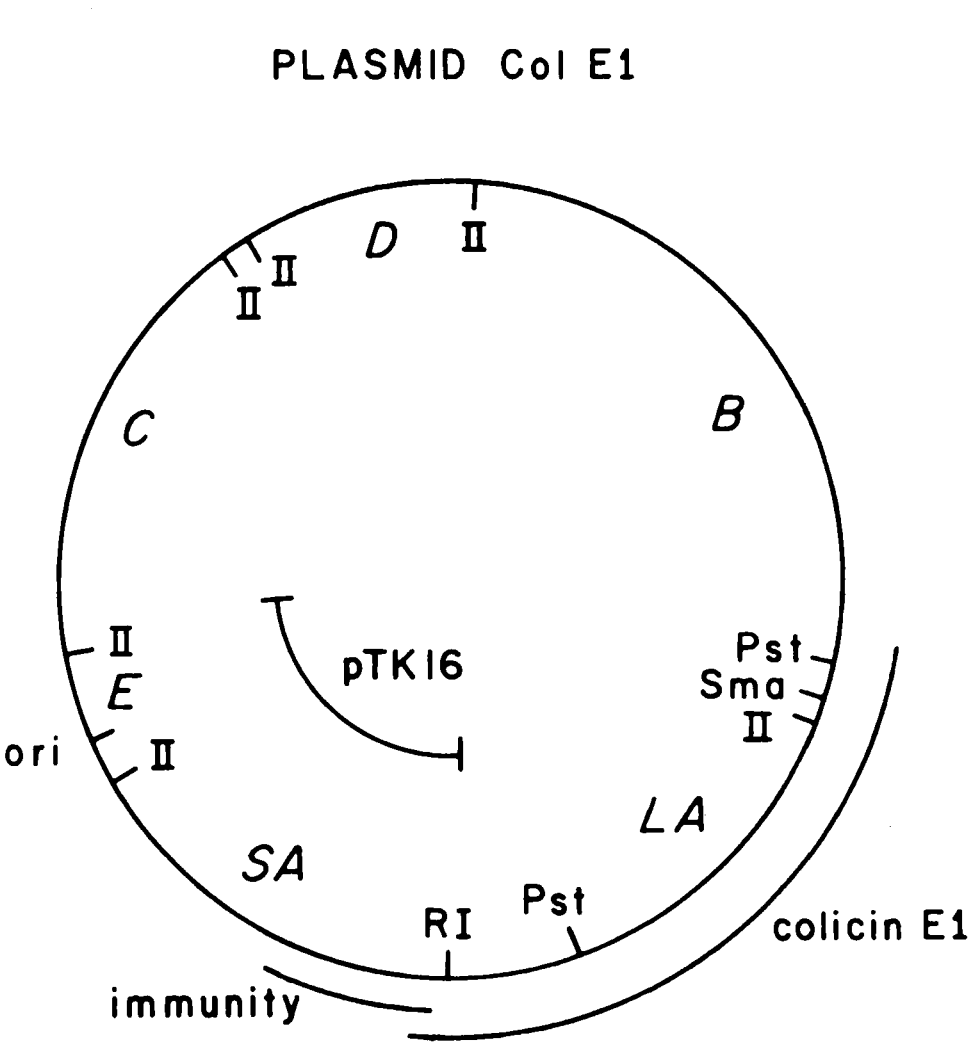

FIGURE 8. Physical and genetic map of plasmid ColE1. Region of ColE1 present in the ColE1 derivative pTK16 is indicated by the inner arc. Mini-ColE1 extends from the EcoRI site clockwise to within fragment D. The Hae II restriction sites, "II", are from Oka and Takanami[45] and unpublished observations (M. Kahn).[47] Colicin El and immunity refer to the genetic regions specifying colicin El production and immunity to this protein, respectively. (Taken from Figurski, D, Kolter, R., Meyer, R., Kahn, M., Eichenlaub, R., and Helinski, D. R., in *Microbiology 1978*, Schlessinger, D., Ed., American Society for Microbiology, Washington, D.C., 1978, 105.)

ColE1 by cleavage of the purified plasmid DNA with the cohesive end-generating restriction endonuclease Hae II. Transformation of *E. coli* cells with a Hae II digest of ColE1 or ColE1 derivatives and ligation of this mixture to a nonreplicating, 1500-base pair Hae II fragment carrying a gene specifying resistance to the antibiotic kanamycin, resulted in the construction of a ColE1 derivative carrying only the Hae II-A and E fragments of ColE1.[47] Further analysis of this low molecular weight ColE1 derivative has indicated that only a portion of the Hae II-A fragment (designated SA) is required in addition to the E fragment for autonomous replication of ColE1. The E and SA fragments (total size, 1300 base pairs) must be joined in the orientation shown in Figure 8 in order to replicate autonomously in *E. coli*.[3]

In the process of constructing low molecular weight derivatives of ColE1, a plasmid, designated pTK16, possessing the E and SA fragments plus the Hae II kanamycin

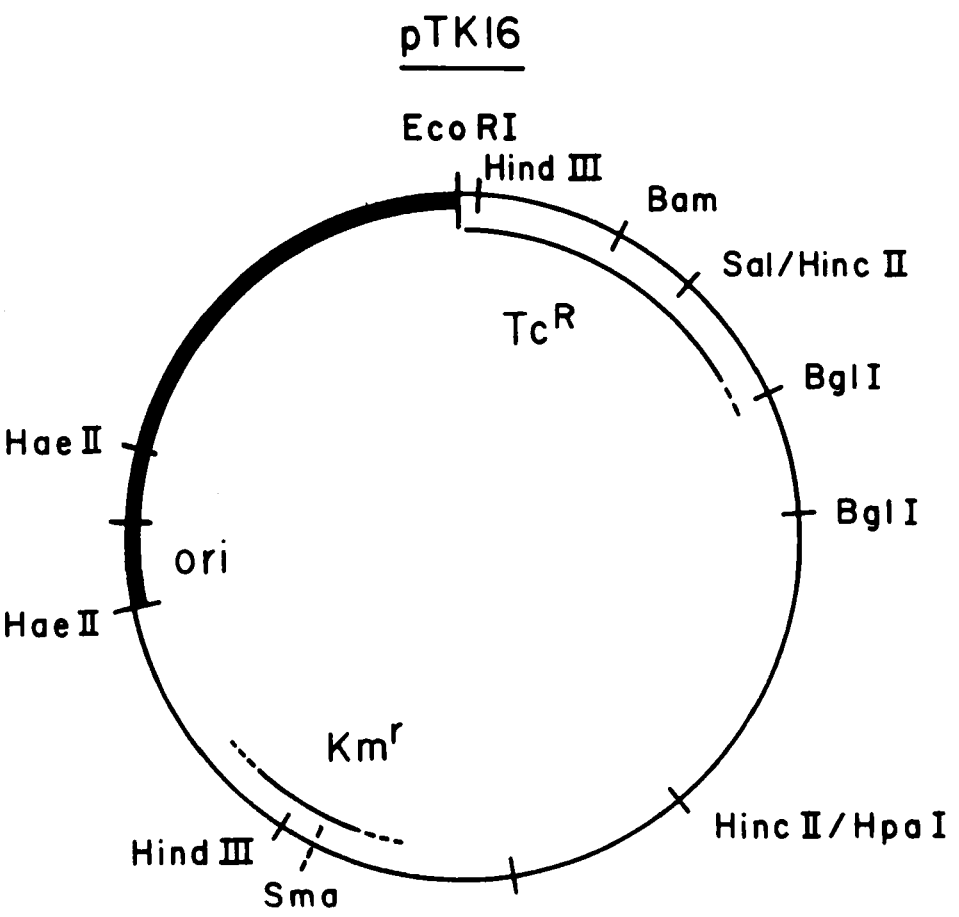

FIGURE 9. Physical and genetic map of the ColE1 derivative, pTK16. Tc^R and Km^R refer to regions specifying resistance to tetracycline and kanamycin, respectively. Positions of sites for a variety of restriction endonucleases are indicated. (Taken from Kahn, M. and Helinski, D. R., manuscript in preparation. With permission.)

resistance fragment of DNA and a segment of DNA carrying the tetracycline resistant gene obtained from plasmid pSC101, was constructed and possesses several features that are advantageous for its use as a plasmid cloning vehicle.[47] Plasmid pTK16 (molecular weight of 2.8×10^6) is maintained as 50 copies per cell and is chloramphenicol amplifiable. The plasmid is poorly mobilizable out of its host cell in the presence of a conjugative plasmid, and as shown in Figure 9, it possesses single sites for several useful restriction enzymes. Insertion of foreign DNA in the Bam HI and Sal I sites results in the inactivation of tetracycline resistance, while insertion at the Sma I and Hind III sites inactivates kanamycin resistance. This insertional inactivation of antibiotic resistance can be made use of in screening bacterial cells that have been transformed for the hybrid form of s plasmid carrying a foreign DNA insert at one of these four restriction enzyme sites.

CONSTRUCTION OF A PHASMID: A PLASMID-PHAGE HYBRID

One of the basic differences between a plasmid element and a bacteriophage is that plasmids are restricted to an intracellular state while bacteriophage particles can exist extracellularly in the form of infectious viral particles. Existence of a plasmid only in

the intracellular state limits experimental approaches to the study in vivo of plasmid replication, incompatibility, and expression. In view of the need for an efficient method of introducing plasmids into cells synchronously and quantitatively, the ColE1 plasmid was reconstructed by recombinant DNA techniques to allow it to be packaged in vivo into bacteriophage particles.[48] A plasmid element packaged in the form of bacteriophage particles can then be injected quantitatively into a suitable recipient bacterial cell. Among other advantages, the construction of a plasmid with bacteriophage properties would allow approaches to plasmid DNA replication that generally have been very successful in the study of bacteriophage DNA replication.

Bacteriphage P4 has several unusual properties that can be used to facilitate the packaging of a plasmid element. P4 is a defective virus in that its lytic growth is dependent on the presence in the bacterial cell of a helper bacteriophage, P2.[49] P4 bacteriophage does not exhibit lytic functions when grown in the absence of P2. P4 bacteriophage, however, can efficiently activate a P2 prophage and produce P4 phage free of P2 helper contamination. The construction by in vitro techniques of a phage-plasmid hybrid DNA molecule made up of a segment of the P4 genome and a low molecular weight derivative of plasmid ColE1 (designated pMK20) (molecular weight of 2.8 $\times 10^6$) is shown in Figure 10. This phage-plasmid, or "phasmid", designated P420, can be readily interconverted between the phage and plasmid states.[48] Infection of *E. coli* cells carrying the P2 helper bacteriophage in the lysogenic state with the P420 hybrid results in replication of P420 DNA and its packaging in the form of infectious particles. Infection of *E. coli* cells nonlysogenic for P2 virus results in stable replication of the P420 hybrid as a plasmid. This unique property of the P420 phasmid has permitted us to ask whether a postive acting protein(s) specified by the ColE1 plasmid is required for replication of the plasmid molecule. This question is particularly relevant with respect to the ColE1 plasmid, since as indicated earlier, ColE1 is unusual in its ability to continue to replicate in *E. coli* cells in the presence of the protein synthesis inhibitor, chloramphenicol. This observation alone does not distinguish between the possibilities that plasmid ColE1 replicates in the absence of a plasmid-encoded protein, or it utilizes under these conditions a plasmid-encoded protein synthesized prior to the addition of chloramphenicol to the growing cells. To resolve this question, *E. coli* cells were infected in the presence of the protein synthesis inhibitors chloramphenicol or puromycin with the P420 hybrid packaged in the form of bacteriophage particles.[48] It was observed that P420 replication, directed by the ColE1 replicon, occurred at a rate equivalent to that found for intact plasmid ColE1 in the presence of chloramphenicol. These results indicate that ColE1 does not require a positive-acting, plasmid-coded protein to replicate its DNA in vivo and provide at least one demonstration of the utility of the P4 bacteriophage system for coupling bacteriophage properties to a plasmid replicon. This finding is consistent with the observed ability of cell-free extracts, prepared from cells that do not carry ColE1, to replicate exogenous ColE1 DNA.[50] These observations also underscore a basic difference between the ColE1 plasmid and most other naturally occurring plasmids that appear to require a plasmid-encoded protein(s) for the regulation of plasmid DNA replication.

DERIVATIVES OF THE BROAD HOST RANGE PLASMID RK2

Studies of the replication properties of low molecular weight derivatives of plasmids ColE1 and Flac described above and similar studies with several other plasmids reported to date[4-9] indicate that in most cases, the essential genes for maintenance of the plasmid in the autonomous state are clustered in a relatively small region of the plasmid molecule. An exception to this general observation is the plasmid RK2; a con-

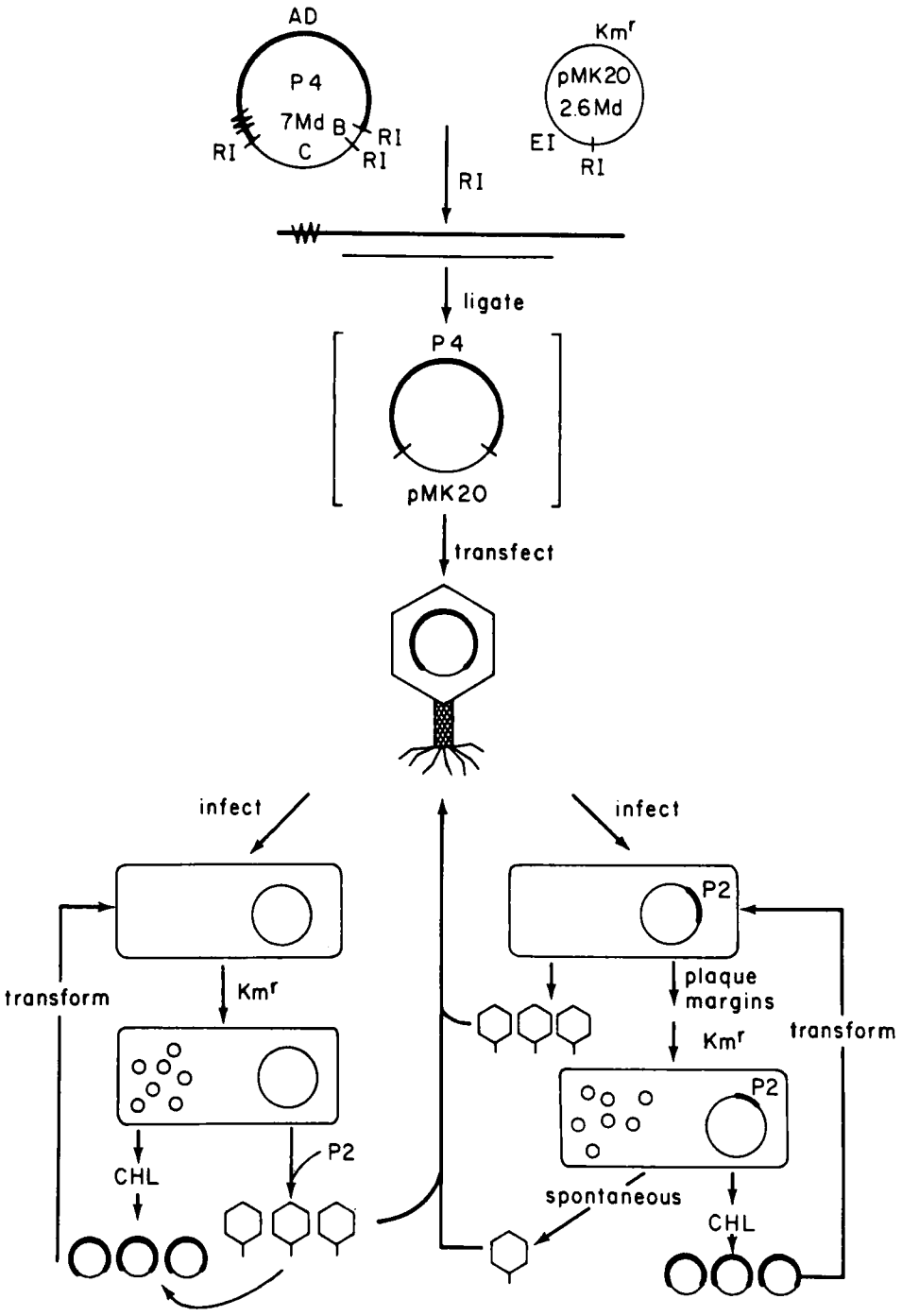

FIGURE 10. Construction and properties of a ColE1-P4 hybrid. Plasmid pMK20 is a derivative of ColE1 that specifies resistance to kanamycin (Km^r). The cos site (ww) of bacteriophage P4 is present on the EcoRI AD fragment. P2 refers to the bacteriophage P2. CHL treatment involves incubation of the cells in the presence of chloramphenicol. (This diagram was prepared by M. Kahn and is based on the data of Kahn and Helinski.[48])

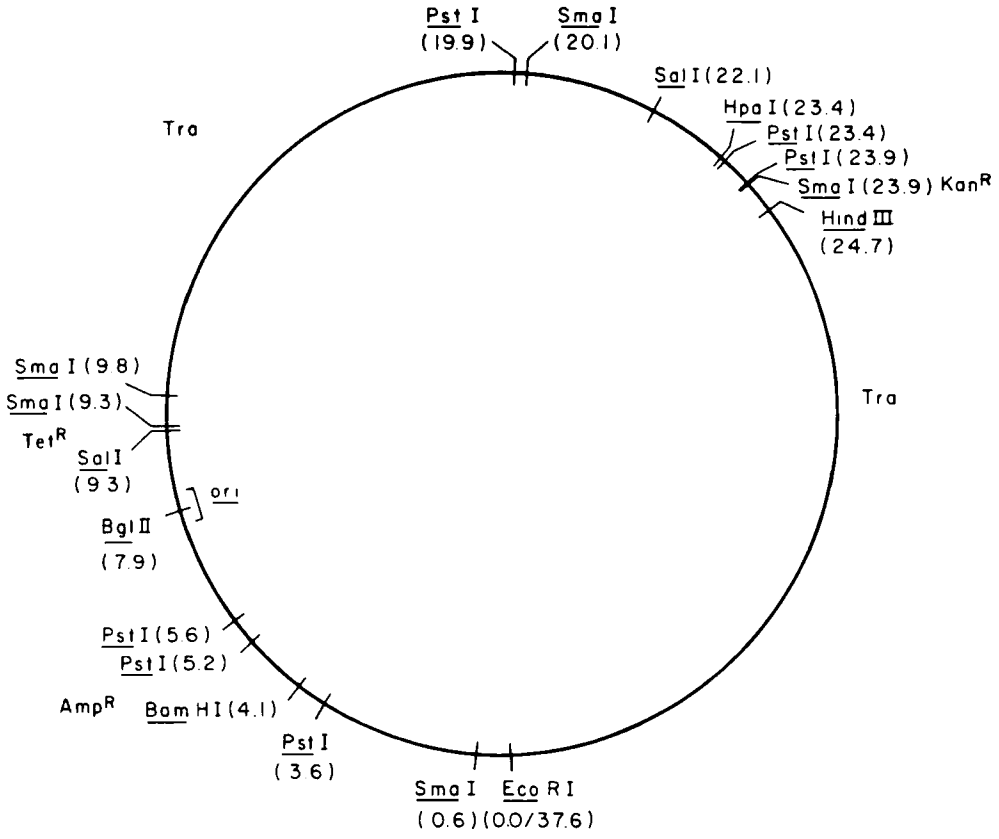

FIGURE 11. Physical and genetic map of plasmid RK2. The regions responsible for self-transmissibility (Tra) and for resistance to kanamycin (Kan^r), ampicillin (Amp^r), and tetracycline (Tet^r) are shown relative to the cleavage sites for various restriction endonucleases. The numbers represent the distance in megadaltons from the EcoRI site. "Ori" refers to the origin of replication. Based on the data of R. Meyer and D. Figurski. (Taken from Meyer, R., Figurski, D., and Helinski, D. R., in *DNA Insertion Elements, Plasmids and Episomes*, Shapiro, J. A. and Adhya, S. L., Eds., Cold Spring Laboratories, Cold Spring Harbor, N.Y., 1977, 680.)

jugative plasmid with a molecular weight of 37.6×10^6 that possesses determinants for resistance to ampicillin, kanamycin, and tetracycline. Unlike mini-F andColE1, RK2 is a member of the P-1 group of plasmids that have an extensive host range among gram-negative bacteria.[20-22] Plasmids of this group are stably maintained in *E. coli*, other members of the Enterobacteriacea family of bacteria and species of agriculturally important genera of Gram-negative bacteria including *Rhizobium, Agrobacterium*, and *Azotobacter*. A project has been initiated in our laboratory to analyze the replication properties of RK2 in the hope of obtaining information on the biochemical and genetic basis for its broad host range and for the purpose of constructing derivatives of plasmid RK2 that may be of use for gene cloning in bacteria distantly related to *E. coli*. A physical and genetic map of plasmid RK2 is shown in Figure 11. It should be noted that despite its relatively large size, RK2 possesses fewer than the expected number of cleavage sites for restriction endonucleases that recognize hexanucleotide sequences. Cleavage sites that are present are most frequently found in regions specifying antibiotic resistance or in locations that are not essential for plasmid replication in *E. coli*. The construction of a detailed restriction endonuclease cleavage map of this plasmid has, as in the case of studies with the other plasmids described above, permitted the use of specific restriction enzymes to delete regions of plasmid RK2 that are not

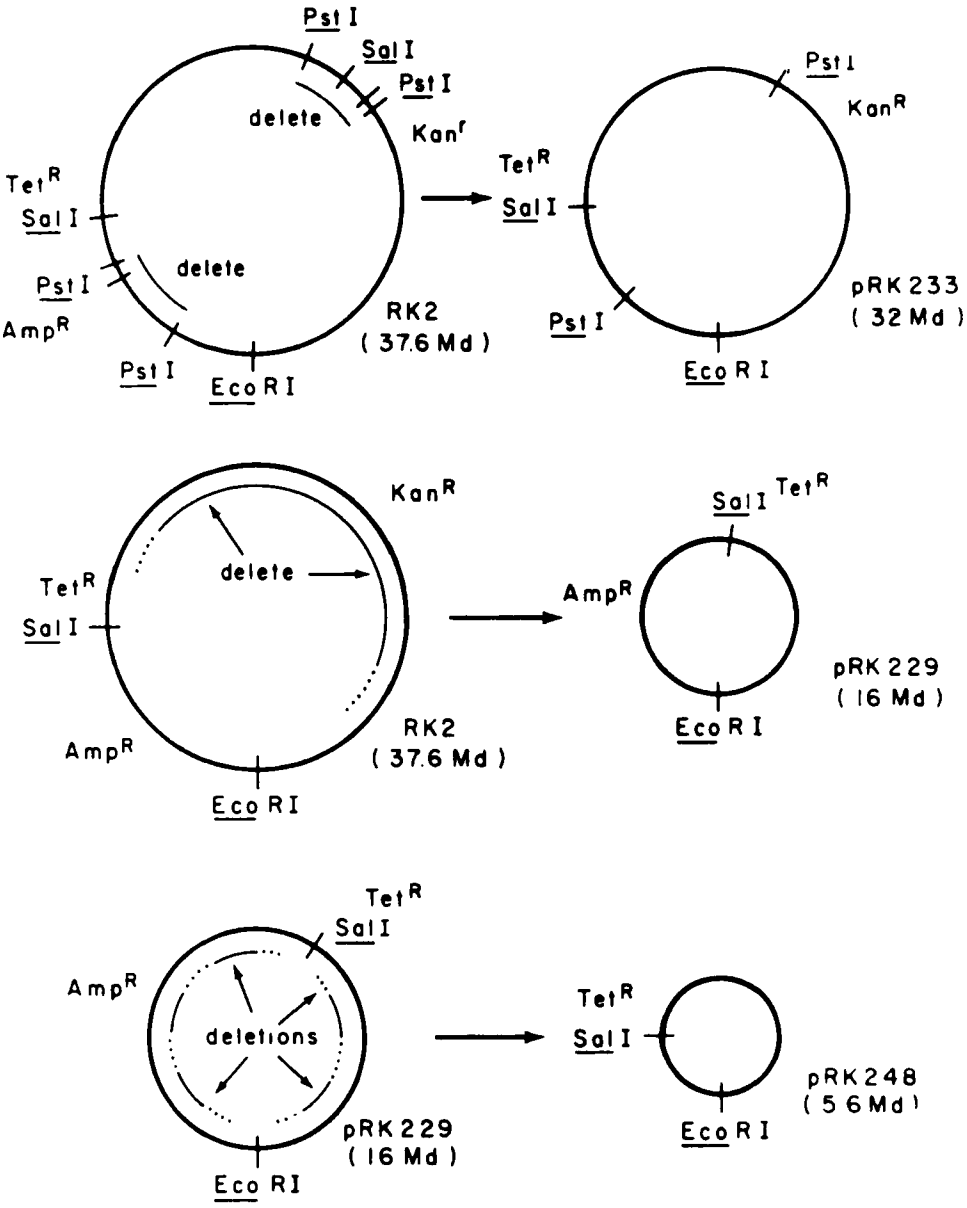

FIGURE 12. Construction of low molecular weight derivatives of plasmid RK2. The construction of the RK2 derivatives was carried out by R. Meyer and is described in the text. (Taken from Meyer, R., Figurski, D., and Helinski, D. R., manuscript in preparation. With permission.)

essential for its replication in *E. coli*. For example, as shown in Figure 12, the restriction enzyme Pst 1 has been successfully employed to delete two nonessential regions of plasmid RK2 leading to the construction of pRK233, a lower molecular weight derivative.[52] Plasmid RK2 is cleaved at a number of sites by the restriction endonuclease Hae II (Hae II sites are not shown in Figure 11). Transformation of *E. coli* cells with a partial Hae II digest of RK2 has resulted in the generation of a plasmid derivative, designated pRK229, that possesses a molecular weight of 16×10^6.[52] As shown in Figure 12, this plasmid has retained the complete small EcoRI-Sal I region of RK2 that includes the origin of replication as well as segments of DNA on either side. It has been

very difficult to further reduce the size of RK2 by the procedures that have been successfully employed for the Flac and ColE1 plasmids, since the genetic regions essential for replication of RK2 in *E. coli* are not clustered and extend over a relatively large portion of the plasmid. It has been possible, however, to further reduce the size of the RK2 plasmid by Hae II cleavage of pRK229 followed by random rearrangement of the fragments and ligation.[52] One relatively small RK2 derivative isolated by this procedure, designated pRK248, is 5.6×10^6 daltons and has retained the genetic region responsible for resistance to tetracycline. Both pRK229 and pRK248 have a copy number similar to that of the parental RK2 plasmid (approximately five copies per chromosome), and both exhibit at least some of the RK2 incompatibility properties.

Plasmid derivatives pRK229 and pRK248 may be particularly suitable as broad host range cloning vehicles in that they are of relatively low molecular weight, contain at least one antibiotic resistance gene for selection of cells transformed for these plasmids, and possess a single sensitive site to at least one restriction endonuclease that generates cohesive ends. In addition, both plasmids are nonconjugative, since the essential genes for conjugation have been deleted in the construction of these plasmids. It should be emphasized, however, that to date the replication of pRK229 and pRK248 has been examined only in *E. coli*, and it is conceivable that a genetic region(s) responsible for the broad host range replication properties of plasmid RK2 was deleted in their construction. If this is the case, while it would be of considerable interest in defining a region(s) responsible for the broad host range properties of a P-1 group plasmid, these plasmid derivatives would be of little use as molecular vehicles for the cloning of genes in bacteria distantly related to *E. coli*.

CONCLUDING REMARKS

From studies carried out in vitro and in vivo, considerable information has been obtained on the genetic and biochemical properties of plasmid elements that occur naturally in *E. coli* and other gram-negative bacteria. The advent of recombinant DNA techniques has provided powerful new approaches for the analysis of factors regulating replication of these plasmids. Information from these new approaches has in turn provided the basis for the in vitro construction of a variety of plasmids that are very useful for the cloning of DNA in *E. coli* and other bacteria. The recent advances in the methods for nucleotide sequence analysis and chemical synthesis of DNA offer exciting new possibilities for studying plasmid DNA replication. The use of these new experimental approaches promises to expand greatly our understanding of the molecular and genetic basis of the extrachromosomal state of DNA in bacteria and, hopefully, will provide new insights into the replication of other circular DNA elements found in bacteria and in eukaryotic cells.

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REFERENCES

1. Timmis, K., Cabello, F., and Cohen, S. N., Cloning, isolation, and characterization of replication regions of complex plasmid genomes, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 2242, 1975.
2. Lovett, M. A. and Helinski, D. R., Method for the isolation of the replication region of a bacterial replicon: construction of a mini F'Km plasmid, *J. Bacteriol.*, 127, 982, 1976.
3. Hershfield, V., Boyer, H. W., Chow, L., and Helinski, D. R., Characterization of a mini-ColE1 plasmid, *J. Bacteriol.*, 126, 447, 1976.
4. Avni, H., Berg, P. E., and Markovitz, A., New mini-ColE1 as a molecular cloning vehicle, *J. Bacteriol.*, 129, 358, 1977.
5. Oka, A., Fine cleavage map of a small Colicin E1 plasmid carrying genes responsible for replication and colicin E1 immunity, *J. Bacteriol.*, 133, 916, 1978.
6. Goebel, W., Blohm, D., Lindenmaier, W., Luibrand, G., and Mayer, H., Characterization of miniplasmids (Rsc) of the R1 drd-19B2 factor carrying the ampicillin transposon, in *Microbiology 1978*, Schlessinger, D., Ed., American Society for Microbiology, Washington, D.C., 1978, 88.
7. Timmis, K. H., Andres, I., Cabello, F., Synenki, R. M., Cohen, S. N., and Burkhardt, H. J., Replication functions of antibiotic resistance plasmid R6-5 and fertility plasmid F'*lac*, in *Microbiology—1978*, Schlessinger, D., Ed., American Society for Microbiology, Washington, D.C., 1978, 99.
8. Figurski, D., Kolter, R., Meyer, R., Kahn, M., Eichenlaub, R., and Helinski, D. R., Replication regions of plasmids ColE1, F, R6K and RK2, in *Microbiology—1978*, Schlessinger, D., Ed., American Society for Microbiology, Washington, D.C., 1978, 105.
9. Crosa, J. H., Luttropp, L. K., Thomas, M. F., and Falkow, S., Replication of R6K and its derivatives in *E. coli* K-12, in *Microbiology—1978*, Schlessinger, D., Ed., American Society for Microbiology, Washington, D.C., 1978, 110.
10. Achtman, M., Willetts, N., and Clark, A. J., A conjugational complementational analysis of transfer-deficient mutants of Flac in *E. coli*, *J. Bacteriol.*, 110, 831, 1972.
11. Sharp, P. A., Hsu, M., Ohtsubo, E., and Davidson, N., Electron microscope heteroduplex studies of sequence relations among plasmids of *E. coli*. I. Structure of F. prime factors, *J. Mol. Biol.*, 71, 471, 1972.
12. Meyer, R. J., Figurski, D., and Helinski, D. R., Properties of the plasmid RK2 as a cloning vehicle, in *DNA Insertion Elements, Plasmids and Episomes*, Bukhari, A. I., Shapiro, J. A., and Adhya, S. L., Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1977, 559.
13. Lovett, M. A., Katz, L., and Helinski, D. R., Unidirectional replication of plasmid ColE1 DNA and relation of the origin-terminus to the nicked site in the ColE1 relaxation complex, *Nature (London)*, 251, 337, 1974.
14. Katz, L., Williams, P. H., Sato, S., Leavitt, R. W., and Helinski, D. R., Purification and characterization of covalently closed replicative intermediates of ColE1 DNA from *E. coli*, *Biochemistry*, 16, 1677, 1977.
15. Fareed, G. C., Garon, C. F., and Salzman, N. P., Origin and direction of simian virus 40 deoxyribonucleic acid replication, *J. Virol.*, 10, 484, 1972.
16. Lovett, M. A., Sparks, R. B., and Helinski, D. R., Bidirectional replication of R6K DNA in *E. coli*. Correspondence between origin of replication and position of single-strand break in relaxed complex, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 2905, 1975.
17. Eichenlaub, R., Figurski, D., and Helinski, D. R., Bidirectional replication from a unique origin in mini F plasmid, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1138, 1977.
18. Meyer, R. J. and Helinski, D. R., Unidirectional replication of the P-group plasmid RK2, *Biochim. Biophys. Acta*, 478, 109, 1977.
19. Jacob, A. E., Shapiro, J. A., Yamamoto, L., Smith, D. I., Cohen, S. N., and Berg, D., Plasmids in *E. coli* and other enteric bacteria, in *DNA Insertion Elements, Plasmids and Episomes*, Bukhari, A. I., Shapiro, J. A., and Adhya, S. L., Eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1977, 607.
20. Datta, N. and Hedges, R. W., Host range of R factors, *J. Gen. Microbiol.*, 70, 453, 1972.
21. Olsen, R. H. and Shipley, P., Host range and properties of *Pseudomonas aeruginosa* R factor R1822, *J. Bacteriol.*, 113, 772, 1973.
22. Hedges, R. W., Jacob, A. E., and Smith, J. T., Properties of an R factor from Bordetella bronchiseptica., *J. Gen. Microbiol.*, 84, 199, 1974.
23. Helinski, D. R., Plasmids as vectors for gene cloning, in *Genetic Engineering for Nitrogen Fixation*, Hollaender, A., Ed., Plenum Press, New York, 19, 1977.
24. Bazaral, M. and Helinski, D. R., Circular DNA forms of colicinogenic factors E1, E2, and E3 from *Escherichia coli*, *J. Mol. Biol.*, 36, 185, 1968.

25. Clewell, D. B. and Helinski, D. R., Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 DNA and protein in *E. coli*, *J. Bacteriol.*, 110, 1135, 1972.
26. Crosa, J. H., Luttropp, L. K., and Falkow, S., Nature of R-factor replication in the presence of chloramphenicol, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 654, 1975.
27. Veltkamp, E., Barendsen, W., and Nijkamp, H. J. J., Influence of protein and RNA synthesis on the replication of the bacteriocinogenic factor CloDF13 in *E. coli* cells and minicells, *J. Bacteriol.*, 118, 165, 1974.
28. Kontomichalou, P., Mitani, M., and Clowes, R. C., Circular R-factor molecules controlling penicillinase synthesis, replicating in *E. coli* under either relaxed or stringent control, *J. Bacteriol.*, 104, 34, 1970.
29. Kline, B. C. and Helinski, D. R., Fl sex factor of *Escherichia coli*. Size and purification in the form of a strand-specific complex of supercoiled DNA and protein, *Biochemistry*, 10, 4975, 1971.
30. Cohen, S. N. and Miller, C. A., Multiple molecular species of circular R factor DNA isolated from *E. coli*, *Nature (London)*, 224, 1273, 1969.
31. Silver, R. P. and Falkow, S., Specific labeling and physical characterization of R factor DNA in *E. coli*, *J. Bacteriol.*, 104, 331, 1970.
32. Meyer, R., Figurski, D., and Helinski, D. R., Physical and genetic studies with restriction endonucleases on the broad host range plasmid RK2, *Mol. Gen. Genet.*, 152, 129, 1977.
33. So, M., Boyer, H. W., Betlach, M., and Falkow, S., Molecular cloning of an *E. coli* plasmid determinant that encodes for the production of heat-stable enterotoxin, *J. Bacteriol.*, 128, 463, 1976.
34. Bazaral, M. and Helinski, D. R., Circular DNA forms of colicinogenic factors E1, E2, and E3 from *Escherichia coli*, *J. Mol. Biol.*, 36, 185, 1968.
35. Clewell, D. B., Nature of ColE1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol, *J. Bacteriol.*, 110, 667, 1972.
36. Hershfield, V., Boyer, H. W., Yanofsky, C., Lovett, M. A., and Helinski, D. R., Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA, *Proc. Natl. Acad. Sci. U.S.A.*, 71, 3455, 1974.
37. Collins, J., Gene cloning with small plasmids, *Curr. Top. Microbiol. Immunol.*, 78, 121, 1977.
38. Gryczan, T. J. and Dubnau, D., Construction and properties of chimeric plasmids in *B. subtilis*, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1428, 1978.
39. Keggins, K. M., Lovett, P. S., and Duvall, E. J., Molecular cloning of genetically active fragments of *Bacillus* DNA in *Bacillus subtilis* and properties of the vector plasmid pVB110, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1423, 1978.
40. Ehrlich, S. D., DNA cloning in *B. subtilis*, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1433, 1978.
41. Figurski, D., Ito, L., and Helinski, D. R., manuscript in preparation.
42. Eichenlaub, R., Figurski, D., and Helinski, D. R., manuscript in preparation.
43. Crosa, J.H., Luttropp, L. K., Heffron, F., and Falkow, S., Two replication initiation sites on R-plasmid DNA, *Mol. Gen. Genet.*, 140, 39, 1975.
44. Palchaudhuri, S. and Maas, W. K., Physical mapping of a DNA sequence common to plasmids of incompatibility group F I, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1190, 1977.
45. Oka, A. and Takanami, M., Cleavage map of colicin E1 plasmid, *Nature (London)*, 264, 193, 1976.
46. Tomizawa, J., Ohmori, H., and Bird, R. E., Origin of colicin E1 plasmid DNA, *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1865, 1977.
47. Kahn, M. and Helinski, D. R., manuscript in preparation.
48. Kahn, M. and Helinski, D. R., Construction of a novel plasmid-phage hybrid: use of the hybrid to demonstrate ColE1 DNA replication *in vivo* in the absence of a ColE1-specified protein, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
49. Six, E. W. and Klug, C., Bacteriophage P4: a satellite virus depending on a helper such as prophage P2, *Virology*, 51, 327, 1973.
50. Tomizawa, J., Sakakibara, Y., and Kakefuda, T., Replication of colicin E1 plasmid DNA added to cell extracts, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 1050, 1975.
51. Meyer, R., Figurski, D., and Helinski, D. R., Restriction enzyme map of RK2, in *DNA Insertion Elements, Plasmids and Episomes*, Shapiro, J. A. and Adhya, S. L., Eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1977, 680.
52. Meyer, R. and Helinski, D. R., manuscript in preparation.