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Cosmids: A type of plasmid gene-cloning vector that is packageable *in vitro* in bacteriophage λ heads

(bacteriophage λ morphogenesis/restriction analysis)

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ABSTRACT **Evidence is presented that ColE1 hybrid plasmids carrying the cohesive-end site (*cos*) of λ can be used as gene cloning vectors in conjunction with the λ *in vitro* packaging system of Hohn and Murray [(1977) *Proc. Natl. Acad. Sci. USA* 74, 3259–3263]. Due to the requirement for a large DNA molecule for efficient packaging, there is a direct selection for hybrids carrying large sections of foreign DNA. The small vector plasmids do not contribute a large background in the transduced population, which is therefore markedly enriched for large hybrid plasmids (over 90%). The efficiency of the *in vitro* packaging system is on the order of 10^5 *hybrid* clones per microgram of foreign DNA for hybrids in the 20–30 million dalton range.**

The mechanism of packaging DNA into the head of *Escherichia coli* bacteriophage λ has been extensively studied through the development of *in vitro* packaging systems (1–4; for review, see ref. 5). These and studies *in vivo* (6) led to the following findings: monomeric circular DNA was not packaged; head-to-tail polymers (concatemers) of the unit-length λ DNA molecules were efficiently packaged if the cohesive-end site (*cos*), substrate for the packaging-dependent cleavage that produces the cohesive ends of mature λ DNA, was 23–33 megadaltons (MDal) apart; and only a small region in the proximity of the cleavage site was required for recognition by the packaging system (7, 8).

This information implies that *cos*-containing plasmids of less than 23 MDal would not be efficiently packaged due to the circular form of their DNA and their size, but that concatemeric derivatives with DNA inserts would be a packaging substrate. The latter DNA structure resembles a ligation mixture between a cleaved *cos*-containing plasmid and DNA to be cloned. It was expected, therefore, that cloning in a *cos*-containing plasmid in conjunction with *in vitro* packaging selects against re-ligated vector molecules but selects for hybrids in the size range of λ DNA, molecules that are recovered only poorly upon transformation.

In our present study, experiments are described in which a *cos*-containing ColE1 *rpo* plasmid (9, 10) was packaged *in vitro* after restriction and re-ligation. The results of this experiment, as well as of RI plasmid and *Pseudomonas* cloning experiments, suggest the use of packageable plasmids as a gene cloning system that is both highly efficient and selective for recovery of large hybrids.

Plasmids containing a *cos* site, which are useful as vectors for gene cloning in conjunction with the packaging system, we refer to as "cosmids."

MATERIALS AND METHODS

Plasmids and Bacteria. Preparation of plasmids pJC720 and pJC703 (Fig. 1) has been described (9, 10). The detailed mapping of these plasmids with restriction endonucleases is unpublished. *E. coli* N205, an *E. coli* K-12 strain ($r_k^+m_k^+$ *recA*$^-$*su*$^-$), was from N. Sternberg; *strain* 5K ($r_k^-m_k^+$ *thr*$^-$*thi*$^-$) was from S. Glover; strain HB101 ($r_k^-m_k^-$ *leu*$^-$ *pro*$^-$*recA*$^-$) was from H. Boyer; and strain GL1 (*pel21*; W3101) was from S. W. Emmons (11).

Packaging System. Exogenous DNA was packaged *in vitro* as described (12), with some slight modifications: single colonies of strains N205 (λimm$_{434}$*cI*$_{ts}$b2 *red*3 *E*am4 Sam7)/λ and N205 (λimm$_{434}$*cI*$_{ts}$b2*red*3 *E*am 15 Sam7/λ were streaked out on LA plates (1) and grown overnight at 30°C. Controls were plated to check temperature sensitivity at 42°C. Single colonies were inoculated into warmed LB medium (1) at an OD_{600} of not more than 0.15 and incubated with shaking until an OD_{600} of 0.3 was reached. Prophages were induced by incubation of the cultures at 45°C for 15 min while standing. Thereafter they were transferred to 37°C and incubated for 3 additional hr with vigorous aeration. (A small sample of each culture, which is lysis-inhibited as a result of the mutation in gene S, was checked for induction: upon addition of a drop of chloroform the culture cleared.) The two cultures were then mixed, centrifuged at 5000 rpm for 10 min, and resuspended at 0°C in 1/500th the original culture volume in complementation buffer (40 mM Tris·HCl, pH 8.0/10 mM spermidine hydrochloride/10 mM putrescine hydrochloride/0.1% mercaptoethanol/7% dimethyl sulfoxide) which was made 1.5 mM in ATP. Biological activity of endogenous DNA can be destroyed by UV irradiation prior to concentration (12). This cell suspension was distributed in 20-μl portions in 1.5-ml Eppendorf polyallomer centrifuge tubes, frozen in liquid N_2, and stored at −60°C. When needed, a sample was transferred in liquid N_2 and put on ice. Immediately on thawing (3–4 min on ice), the DNA to be packaged (0.01–0.2 μg) was added in a volume of 1–5 μl. The DNA was usually added in the buffer in which it had just been ligated. The solutions were carefully mixed and bubbles were removed by a few seconds' centrifugation in an Eppendorf desk-top centrifuge. The mix was incubated for 30 min at 37°C. At the end of this incubation period, 20 μl of a frozen and thawed packaging mixture, which had been made 10 mM in $MgCl_2$ and to which a final 10 μg of DNase per ml was added, was mixed to each sample and incubation was continued for 20–60 min. SMC (1) buffer (0.5 ml) and a drop of chloroform were added. After mixing, denatured material was centrifuged off and the solution was used as a phage lysate.

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Abbreviations: MDal, megadaltons; cosmid, plasmid containing a *cos* site.

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Transduction was carried out by adding 0.4 ml of this phage suspension to 1 ml of N205 or *pel*$^-$ cells from a late exponential culture (OD_{600} = 2.0) in L broth–maltose [1% Bacto-Tryptone/0.5% yeast extract (Oxoid)/0.5% NaCl/0.4% maltose]. For the experiment with *Pseudomonas* DNA, HB101 was used as recipient. After a 10-min adsorption at 30°C, the mixture was diluted 1:20 in fresh L broth and incubated for 2 hr at 30°C to allow expression of rifampicin resistance.

Transformation. Strain 5K was used. Cultures grown to an OD_{600} of 0.5 were cooled rapidly on ice, centrifuged, and resuspended in 0.5 vol of 10 mM NaCl on ice. After 30 min on ice, the cells were centrifuged and resuspended in 0.5 vol of 50 mM $CaCl_2$ and again incubated for 30 min at 0°C. After centrifugation, the cells were resuspended in 0.1 vol of 30 mM $CaCl_2$ in 20% glycerol. This competent cell preparation, divided into 1-ml aliquots, was kept frozen at −60°C until needed. For transformation the sample was thawed out on ice and 0.5 ml of 40 mM Tris, pH 8.0/40 mM NaCl/1 mM EDTA, containing the DNA for the transformation (0.1–1 μg), was added. After 30 min on ice, the mixture was heated to 42°C for 2 min and rapidly cooled on ice. The cells were diluted 1:30 in L broth and incubated for 2 hr at 37°C to allow expression of rifampicin resistance (13).

Rifampicin resistance was tested on L broth plates containing either 100 μg of rifampicin per ml, when plasmid pJC703 was used, or 30 μg/ml, when plasmid pJC720 was used. The colonies derived after transduction or transformation of pJC720 grow slowly on rifampicin, taking 2 days at 37°C to form large colonies. Since rifampicin is light sensitive, the plates must be kept dark during this prolonged incubation to prevent growth of background colonies.

Restriction and Ligation Reactions. Restriction with *Hin*dIII (Boehringer) was carried out in 30 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/10 mM NaCl to completion. Digestion with *Sal* I, *Eco*RI, and *Bgl* II was carried out in the same buffer. *Sal* I and *Eco*RI were generous gifts from H. Mayer and H. Schütte, and *Bgl* II was a generous gift from E. Eichenlaub. Digestion with *Kpn* I (Bio-Labs) was in 10 mM Tris·HCl, pH 7.9/6 mM $MgCl_2$/6 mM NaCl/10 mM dithiothreitol containing 100 μg of bovine serum albumin per ml. Gel electrophoresis was in 1% agarose (14).

Ligation was carried out after heat inactivation of the restriction endonucleases at 70°C for 10 min in 6 mM $MgCl_2$, 10 mM Tris·HCl (pH 7.9), 10 mM dithiothreitol, 100 μM ATP, 100 μg of bovine serum albumin per ml, and 5×10^{-2} unit of T4 DNA ligase (Boehringer) in 100-μl aliquots. The concentration of DNA ends was between 20 and 60 pM or as described in the text. Before the ligase was added the samples were mixed, heated to 70°C for 5 min, and cooled on ice for 30 min. The ligation reaction was continued for 15 hr at 8°C. Completion of the ligation was checked by agarose gel electrophoresis: no sample was used in which linear monomers could still be detected.

Nomenclature of Plasmids. The numbering of the plasmids in the pJC series coming from John Collins will be confined to the first 500 in each thousand, with the exception of those already published, so as to avoid confusion with the collection of Alvin John Clark; pJC703 and pJC720 are two of these exceptions.

Safety Regulations. All experiments described here were carried out under P1 conditions as defined by the National Institutes of Health guidelines for recombinant DNA research.

RESULTS

Packaging of restricted and re-ligated plasmid DNA

Production of Packageable Substrate from Plasmid DNA. Plasmid pJC703 (Fig. 1) yields two *Hin*dIII restriction fragments: the 10-MDal fragment A containing the λ *cos* site and the ColE1 replicon, and the 7-MDal fragment B containing the gene for rifampicin resistance (*rpoB*). It was hoped that cleavage and re-ligation of plasmid pJC703 would produce a population of polymers (some of which are diagrammed in Fig.

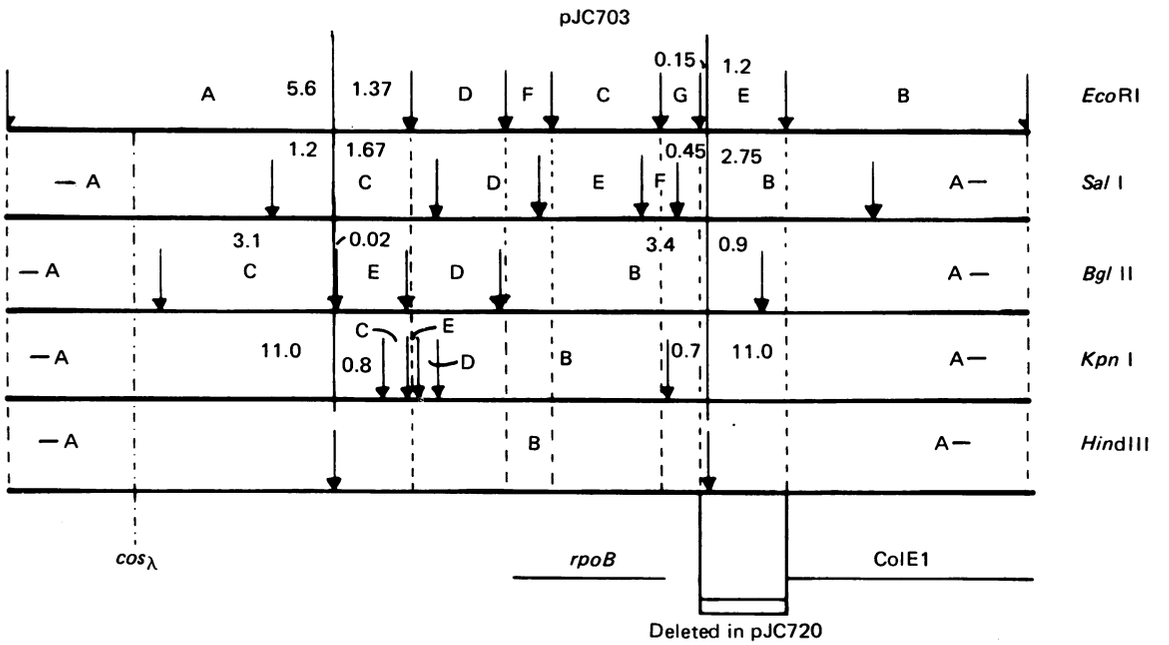

FIG. 1. Restriction endonuclease map of pJC703 and pJC720. Fragments obtained with each enzyme are alphabetically labeled according to size. Dashed lines indicate the relative positions of the *Eco*RI cleavage site on each map, and the continuous vertical lines the positions of the *Hin*dIII cleavage sites. The distances from the *Hin*dIII sites to the nearest cleavage sites for each restriction enzyme are indicated in MDal. These values are used in the analysis of plasmids containing polymeric *Hin*dIII fragments (Table 2). The ColE1 part of these plasmids is actually derived from a freak isolate (pJC309) which contains a *Sal* I site not present in ColE1.

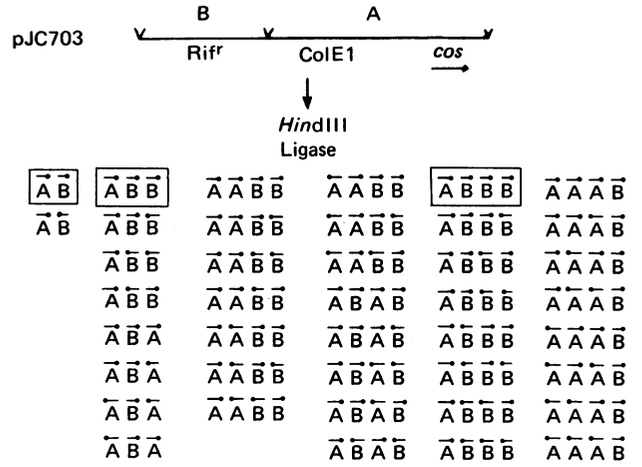

FIG. 2. Cleavage with *Hin*dIII and re-ligation of the two *Hin*dIII fragments (A and B) of plasmid pJC703 theoretically can yield a series of polymers. The diagram shows all the possible cyclic permutations that contain at least one A *and* one B fragment, up to the tetrameric forms. The relative orientations are indicated by the small "matchsticks" over each fragment designation. Those structures actually obtained are framed.

2) that could mimic the natural substrate for packaging. Cleavage of such molecules at the *cos* site during packaging, and recircularization subsequent to transduction, would lead to the loss of one or more entire A fragments, thus generating plasmids of the form ABB, ABBB, and AAB (or AB).

After the re-ligated *Hin*dIII fragments of pJC703 were packaged, several thousand rifampicin-resistant clones were obtained by transduction into N205. The yield of Rif^r clones is dependent on the concentration of the vector DNA during the ligation (Exp. 1 *a* and *b*, Table 1). This supports the hypothesis that efficient packaging is dependent on the formation of long polymers. In contrast, the formation of such highly polymerized chains is most detrimental to the efficiency of transformation, as has been noted elsewhere (14). The transformation data are included merely as an additional test that the ligation was successful rather than as a direct comparison of packaging and transformation, since the ligation conditions are strongly biased in favor of packaging experiments.

Structure of Packaged Plasmids. Fifty-two colonies were picked at random for further testing. They were all found to be colicin E1 resistant and colicin E2 sensitive, indicative that the plasmid coded E1-immunity carried on the *Hin*dIII A fragment was present. Small cleared lysates were made from each clone. To check for the presence and approximate size of the plasmid DNA, we electrophoresed 5-μl samples (plus 0.1% sodium dodecyl sulfate) on 0.8% agarose gels (14). Supercoiled DNA was prepared from the first 12 samples and, from the remaining 40, from those showing the presence of plasmids larger than pJC703. These DNAs (Exp. 2, Table 1), and in some cases the products of a second packaging step, were analyzed more thoroughly with the restriction enzymes *Bgl* II, *Kpn* I, *Sal* I, *Eco*RI, and *Hin*dIII (Fig. 3).

Three size classes of plasmid were obtained: 17 MDal, corresponding to the starting plasmid, and about 23 MDal and 29 MDal. To test the orientation of the fragments with respect to one another, we cleaved the plasmids with an enzyme other than *Hin*dIII. This would generate fragments overlapping the *Hin*dIII junctions and yield "junctional" fragments diagnostic of the *Hin*dIII fragment arrangement (Table 2 and Fig. 3).

Only a small number of the possible structures (Fig. 2) are found amongst the large number analyzed: $\vec{A}\vec{B}$, $\vec{A}\vec{B}\vec{B}$, and $\vec{A}\vec{B}\vec{B}\vec{B}$ (Table 2 and Fig. 3). Absent are plasmids containing duplicates of the *Hin*dIII A fragment, i.e., that fragment carrying the replicon origin and the *cos* site. Such structures would be eliminated if every *cos* site were cleaved during *in vitro* packaging. The *in vivo* probability, however, of cutting a pair of *cos* sites decreases as the amount of DNA between them decreases (6). The existence of plasmids having tandem ColE1 origins (unpublished observation) would support the argument that there is no *a priori* reason to expect plasmids containing two A fragments to be unstable. The absence of the double A combination is therefore not easily explained.

The absence of the opposed orientation of the AB fragments, namely, $\vec{A}\overleftarrow{B}$, may be due to the dependence of expression of the Rif^r *rpoB* gene on readthrough transcription from the A fragment in the correct orientation (9).

The absence of all palindromic structures (perfect inverted repeats extending to the axis of the symmetry) is also remarkable, as is the high number of deletions found (four from 52 isolates). Whether or not the elimination of palindromic

Table 1. Transformation and packaging efficiencies of plasmids containing *cos* sites, before and after cleavage with *Hin*dIII and ligation with DNA ligase

Exp.	DNA		MDal of vector	DNA, μg/ml	Transformation: Rif^r colonies/ μg DNA	Transformation: % hybrids	Packaging: Rif^r colonies/ μg DNA	Packaging: % hybrids
1*a*	pJC703 × *Hin*dIII, ligated		17	500	30	NT	1.0×10^5	~50
1*b*	pJC703 × *Hin*dIII, ligated		17	50	1.4×10^3	NT	1.0×10^2	
2	Supercoils of pJC703,							
	configuration AB		17		1.4×10^5		1×10^2	
	ABB		23		1.7×10^4		4×10^3	
	ABBB		29		1.8×10^4		1×10^4	
3	pJC720 × *Hin*dIII + RI*drd*19 × *Hin*dIII	Ligated	16	146 + 17	4.1×10^2	0.15	2.4×10^3	~90
4	pJC720 × *Hin*dIII + *Pseudomonas* AM1 × *Hin*dIII	Ligated	16	330 + 75	NT		5×10^3	~80

Transformants or (subsequent to packaging) transductants were selected for on media containing rifampicin. Yields are given as Rif^r colonies per μg of input (vector and foreign) DNA. In Exp. 1, the percentage hybrid clones refers to the percent containing more than one copy of the *Hin*dIII B fragment. About 90% of the Rif^r colonies from Exp. 3 also contained the 11.5-MDal *Hin*dIII fragment from RI*drd*19, which carries ampicillin resistance. The efficiency of packaging λb2 DNA in parallel experiments was about 10^7–10^8 plaque-forming units per μg of input DNA. NT, not tested.

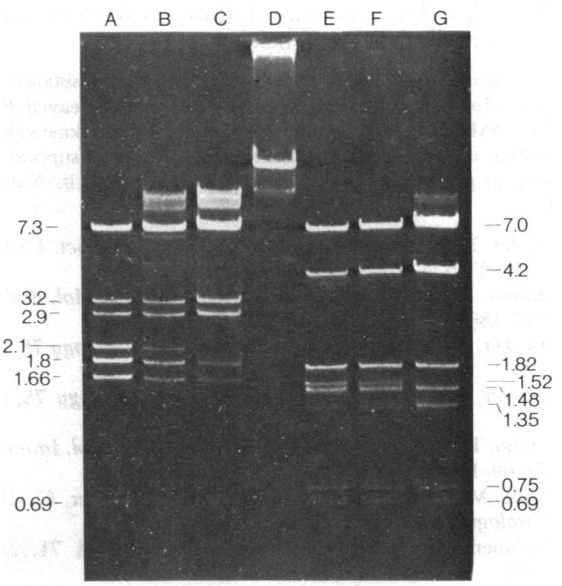

FIG. 3. Agarose gel (1%) electrophoresis of products of restriction endonuclease digestions. (Gels A, B, and C) Digests of *Sal* I endonuclease; (gels E, F, and G) digests of *Eco*RI; (gel D) uncut supercoiled pJC703. Gels A and E contain digests of a 29-MDal isolate derived from Exp. 2 in Table 1; B and F contain digests of a 23-MDal plasmid from the same experiment; C and G contain digests of pJC703. MDal are indicated.

structures, which are certainly present in the ligation mixture, takes place during the packaging step or after the transduction is not known.

Size Selectivity of Packaging. Of the 52 Rif^r clones from the experiment of packaging *Hin*dIII cleaved and re-ligated pJC703 DNA, 14 were of the 23-MDal ABB class, 7 were of the 29-MDal ABBB class, 4 were of intermediate sizes showing also aberrant fragments indicating deletions, and 27 were indistinguishable in size from pJC703. Of these latter, five were tested by restriction enzyme analysis and appeared identical to pJC703.

An even stronger size selection was obtained when Exp. 1 (Table 1) was repeated with a *pel*$^-$ host as recipient. The *pel*$^-$ mutation increases the DNA size dependency of DNA injection by lambdoid bacteriophages (15). From 29 Rif^r *pel*$^-$ clones tested, 19 had plasmids in the 24- to 25-MDal range and 8 in the 29- to 30-MDal range, with a single plasmid of 17 MDal. With the exception of three clones that had small deletions at the junction of the tandemly repeated B fragment and the A fragment, all of the larger plasmids were found to be of the form $\vec{A}\vec{B}\vec{B}$ or $\vec{A}\vec{B}\vec{B}\vec{B}$.

Table 2. Detection of different possible molecular forms in plasmids containing polymeric regions

Molecular form	Junctional fragments: Expected (MDal) *Sal* I	*Eco*RI	Found
$\vec{A}\vec{A}$	3.95	6.8	−
$\overleftarrow{A}\vec{A}$	5.5	2.4	−
$\overleftarrow{A}\overleftarrow{A}$	2.4	11.2	−
$\vec{A}\vec{B}$	3.2	1.35	+
$\vec{A}\overleftarrow{B}$	4.42	2.6	−
$\overleftarrow{A}\overleftarrow{B}$	1.65	5.7	−
$\vec{B}\vec{B}$	2.12	1.52	+
$\vec{B}\overleftarrow{B}$	3.34	2.74	−
$\overleftarrow{B}\overleftarrow{B}$	0.9	0.3	−

Plasmids containing the molecular form indicated (after the convention adopted for Fig. 2) would produce "junctional" fragments of the indicated MDal.

Considering A as a cosmid vector molecule and B as foreign DNA, Exp. 1 (Table 1) can be taken as a model cloning situation. The optimum yield of "hybrid" clones in this experiment would therefore be 3×10^5 per μg of the (foreign) B fragment.

Packaging of Supercoiled DNA. Packaging of supercoiled DNAs of the plasmids (Exp. 2, Table 1) is several orders of magnitude lower than packaging of λ DNA (10^7–10^8 plaque-forming units/μg of λ DNA in parallel experiments), the efficiency for the smallest plasmid being the lowest. Moreover, their structure appears to be unaltered by the packaging-transduction step, as shown by restriction analysis of the supercoiled plasmids isolated from the transductants. Earlier studies on *in vivo* and *in vitro* packaging (summarized in ref. 5) led to the conclusion that supercoiled DNA with a single *cos* site is not packageable without a recombination step, although a low level of *in vivo* packaging of monomeric circular DNA has recently been reported (16). We do not known whether or not the low level of *in vitro* packaging of circular DNA is dependent on a low level of dimer or higher multimers in the supercoiled preparation.

Cloning of RI*drd*19 DNA

The high percentage of larger hybrids in Exp. 1 (Table 1) is evidence that a size selection is occurring in the packaging of cosmid–hybrid DNA. This size dependency was further tested by using the cosmid pJC720 (16 MDal), which contains a single *Hin*dIII site (Fig. 1), to clone fragments from the R factor RI*drd*19 (Exp. 3, Table 1). The *Hin*dIII fragments generated from this plasmid are 42.8, 11.5, 2.9, 2.0, 1.95, 1.8, 0.15, and 0.1 MDal (17). The 11.5-MDal fragment carries the gene for ampicillin resistance.

Of the Rif^r clones obtained after packaging, 90% were also found to be ampicillin resistant and therefore to be carrying at least the 11.5-MDal *Hin*dIII fragment from RI*drd*19. It would seem, therefore, that a very strong size selection had been imposed by packaging, in which the 27.5-MDal (to 30-MDal?) hybrids were produced in preference to the 16- to 19-MDal plasmids. In Exp. 3, the yield is about 2×10^4 *hybrid* clones per μg of *foreign* DNA, even though 80% of the fragments in this mixture were probably either too small (0.1–2.9 MDal) or too large (42.8 MDal) to be efficiently transduced by this method. The efficiency of packaging hybrid cosmids is therefore on the order of 10^5 per μg of foreign DNA of the correct length.

Transformation with the same DNA yielded few Rif^r Amp^r hybrids, the overall transformation efficiency being low due to the high DNA concentration used during ligation.

Cloning of *Pseudomonas* DNA

pJC720 was used to clone fragments from *Pseudomonas* AM1 chromosomal DNA partially digested with *Hin*dIII (Exp. 4, Table 1). By gel electrophoresis it was estimated that more than 80% of the *Pseudomonas* fragments were larger than 16 MDal and probably too large to be clonable by packaging with this vector. In spite of this, the efficiency of *hybrid* formation is about 3×10^4 per μg of foreign DNA. Twenty-seven of the first 32 clones tested carried new DNA fragments. The average size of the DNA insert in these 27 was 10 MDal. On this basis, a few hundred of the clones obtained should constitute a gene bank (18) of *Pseudomonas* AM1 chromosomal DNA in *E. coli*.

DISCUSSION

We have demonstrated that the packaging of plasmid DNA in λ bacteriophage particles can be used as a method for obtaining plasmid hybrids in the 20- to 30-MDal size range when using plasmid DNA that has been linked *in vitro* to foreign DNA fragments. The yield of clones containing these hybrids is of the order of 3×10^5, under optimal conditions, per μg of foreign DNA. Furthermore, by the use of small plasmids (less than 8 MDal) that are themselves very inefficiently packaged (unpublished results), the background of nonhybrid clones is effectively eliminated in a single step without resort to either modification of the DNA (e.g., alkaline phosphatase treatment or polynucleotide tailing) or to elaborate selection or screening procedures which are usually the most time-consuming steps in plasmid cloning experiments. In addition, the use of small cosmids will allow efficient recovery of cloned fragments in the size of up to 25–30 MDal, the selection being imposed by the requirement for packaging of a full or nearly full head.

In vitro packaging of λ cloning vectors can be made independent (12) or dependent (19) of the size of the DNA in the range of 24–30 MDal, but a lower size limit for the vector is set by the requirement for the bacteriophage genes for plaque formation. This requirement is circumvented in the cosmid cloning system, which is independent of phage genes responsible for lytic growth. The space thus provided can be taken up by DNA to be cloned.

Because of the small region required for plasmid replication it is to be expected that new derivatives for use with other restriction enzymes will be rapidly developed. Cosmid derivatives of pJC720 and pJC703 have been produced in which cloning with *Bgl* II or *Bam*HI can be carried out by using rifampicin selection, with *Sal* I, *Eco*RI, *Bgl* II, or *Bam*HI by using ampicillin selection, or with *Xma* I, *Kpn* I, and *Pst* I by using selection for colicin immunity (unpublished results). In addition, a series of cosmid vectors have been developed (unpublished results), including an 8-MDal cosmid (pJC75-58) for use with *Eco*RI, *Bam*HI, and *Bgl* II which is temperature sensitive, ampicillin resistant, and mobilization-minus. It is hoped that in conjunction with incapacitated host strains (20) this latter cosmid will provide an EKII host-vector system that will be most effective in the production of gene banks of eukaryotic DNA.

The packaging of cosmids in λ particles should allow the use of many standard genetic tricks, previously only applicable to λ, for the selection of deletions or insertions in cloned fragments. Such selection methods are based on the instability of full bacteriophage heads in chelating agents, the positive selection for large molecules on infection of *pel*$^-$ hosts, or the physical separations possible on the basis of density differences between full and partially filled λ particles.

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