Charon Phages: Safer Derivatives of Bacteriophage Lambda for DNA Cloning

Abstract. *The Charon λ bacteriophages have been developed as vectors for cloning. Their construction incorporates mutations that make them simple to use and also greatly increases their safety for the biological containment of cloned recombinant DNA. Three of the Charon vector phages, 3A, 4A, and 16A, have been certified for use as EK2 vector-host systems, when propagated in bulk in a special bacterial host, DP50SupF. We present here some of the data on which the safety of these systems was evaluated. DNA fragments ranging in size from 0 to 2.2 × 10^4 base pairs can be cloned in these EK2 Charon phages.*

The desirability of using vectors for DNA cloning which have poor survival in natural environments and which require special conditions for their replication has been amply emphasized in the recent past (*1*, *2*). From the point of view of containment, DNA cloning with lytic phages offers a natural advantage, since two components—phage and sensitive bacteria—must repeatedly come together for significant replication to take place. Phage vectors are also convenient because the chimeric DNA is delivered to the experimenter in a package. Since the phage and bacteria coexist only briefly, a cloned segment need not be compatible with *Escherichia coli* metabolism for extended periods of time.

Bacteriophage λ is particularly well suited for adaptation to make it useful as a lytic cloning vehicle. This is because one-third of its DNA, which forms a continuous block in the middle of the genome, can be replaced without the phage losing its ability to grow lytically (Fig. 1). Several groups of investigators, including our own, have constructed derivatives of λ specifically adapted for DNA cloning (*3*–*5*). The aim in all these adaptations is to construct phages so that a restriction endonuclease can make a cut (or cuts) only in the dispensable one-third of the λ DNA to permit subsequent addition of (or replacement by) a foreign DNA segment. Point mutations, substitutions, and deletions are, therefore, introduced into wild-type λ to alter the distribution of restriction sites and to eliminate sites in the essential regions of the phage genome. Other objectives of vector engineering include (i) cloning a variety of sizes of DNA fragments; (ii) cloning with more than one restriction enzyme; (iii) indicating by plaque type whether or not a phage has incorporated a DNA fragment; (iv) cloning with minimal manipulations; (v) controlling transcription of the cloned fragment from promoters on the vector; (vi) growing vectors and clones to a high

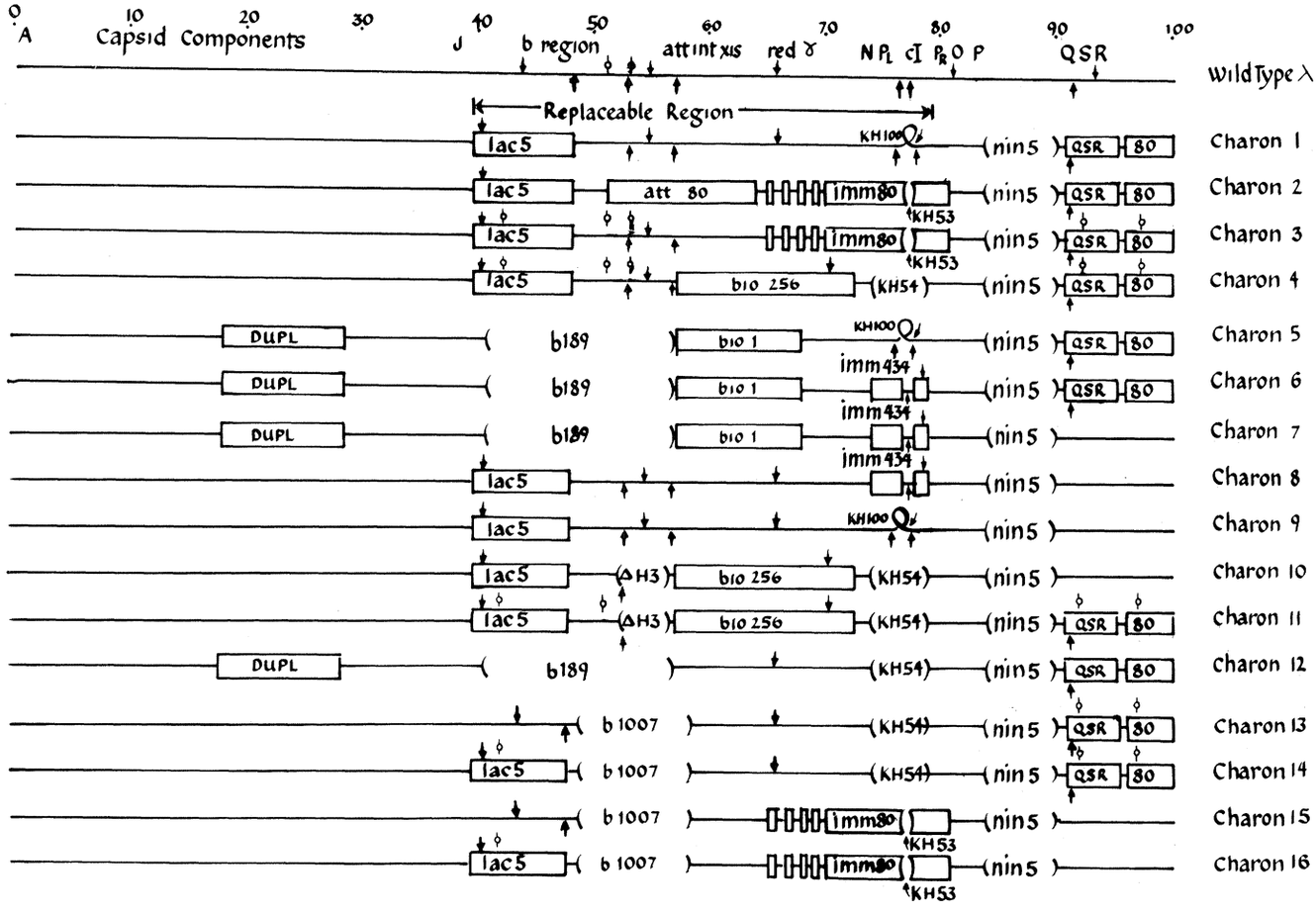


Fig. 1. Physical map of λ and the Charon phages. The phage λ map which we now estimate to be 49,400 ± 600 (S.E.M.) base pairs (*34*) is drawn to scale with the replaceable region indicated relative to essential genes for lytic growth. Beneath it are the Charon phage vectors with portions from λ (indicated by lines) aligned with the λ map. Boxes indicate substitutions, but the lengths of DNA substituted are not shown to scale. The length of DNA in each vector is shown in Table 1. Downward and upward arrows are Eco RI and Hind III sites, respectively. The φ symbol shows known Sst I sites; Charon 15 has none. (Sst I sites were not determined for phages 1, 5, 6, 7, 8, 9, 10, and 12.) DUPL is a duplicated piece of phage λ. *lac*5, *bio*1 and *bio*256 are substitutions from Lac and Bio regions of *E. coli*. The boxes labeled *att*80, *imm*80, *QSR*80 are portions of phage φ80. The region shown by four small boxes at around 70 in some phages is from φ80; it is partially homologous to λ. The parenthesized b189, b1007, ΔH3, KH53, KH54, and *nin*5 are deletions; KH100 is an insertion; *imm*434 is a replacement for immλ [see (*6*)].

yield; (vii) readily recovering cloned DNA; and (viii) features contributing to biological containment.

The λ vectors that we have developed during the past 3 years have been designated Charon phages, after the mythological boatsman of the River Styx. They have been numbered sequentially as constructed. The structures of Charon phages 1 to 16 are shown in Fig. 1. The properties that make them useful as vectors are summarized in Table 1.

Construction of Charon phages. Most of the mutations that were incorporated into Charon phages have been described by us (*6*) and others (*7*). These were assembled into the desired arrangements by standard genetic crosses (*8*). We describe here only those mutations that were isolated for this project and have not previously been described.

The deletion KH53 in the immunity region of φ80 was isolated by selecting for elimination of the restriction endonuclease Eco RI site in the φ80 immunity region according to the method of Arber and Kühnlein (*9*). Concomitant with loss of the restriction site, the plaque morphology changed from turbid to clear. Examination of heteroduplexes (Fig. 2k) between the DNA of KH53 and of a phage with the wild-type φ80 immunity region revealed a single-stranded loop of approximately 750 base pairs near the position of the restriction site. KH53 was shown by measuring its buoyant density to be a deletion rather than an insertion; it probably deletes part of the φ80 analog of the λ *c*I gene. However, since little is known about the genetic organization of the immunity region of φ80, other explanations are possible. As a practical matter, KH53 is nonrevertible and drastically reduces the formation of lysogens (see safety tests).

We selected two mutations, BW1 and BW2, which eliminated the Eco RI sites just to the right of the immunity regions of λ and φ80, respectively. Another mutation isolated for this study is the duplication (DUPL) located on the left arm of Charons 5, 6, 7, and 12. This appears to be similar to duplications described by Bellett *et al.* (*10*) and has been useful for increasing the size of that arm.

The no-cut right end (NRE) of Charons 7, 8, 9, 10, 15, and 16 was specially constructed by combining the *nin*5 deletion with a hybrid isolated by Murray and Murray (*5*) which removes the Hind III site near gene *Q*, and then selecting a new point mutation (DK1) that eliminates the Eco RI site near gene *S*. The NRE, unlike

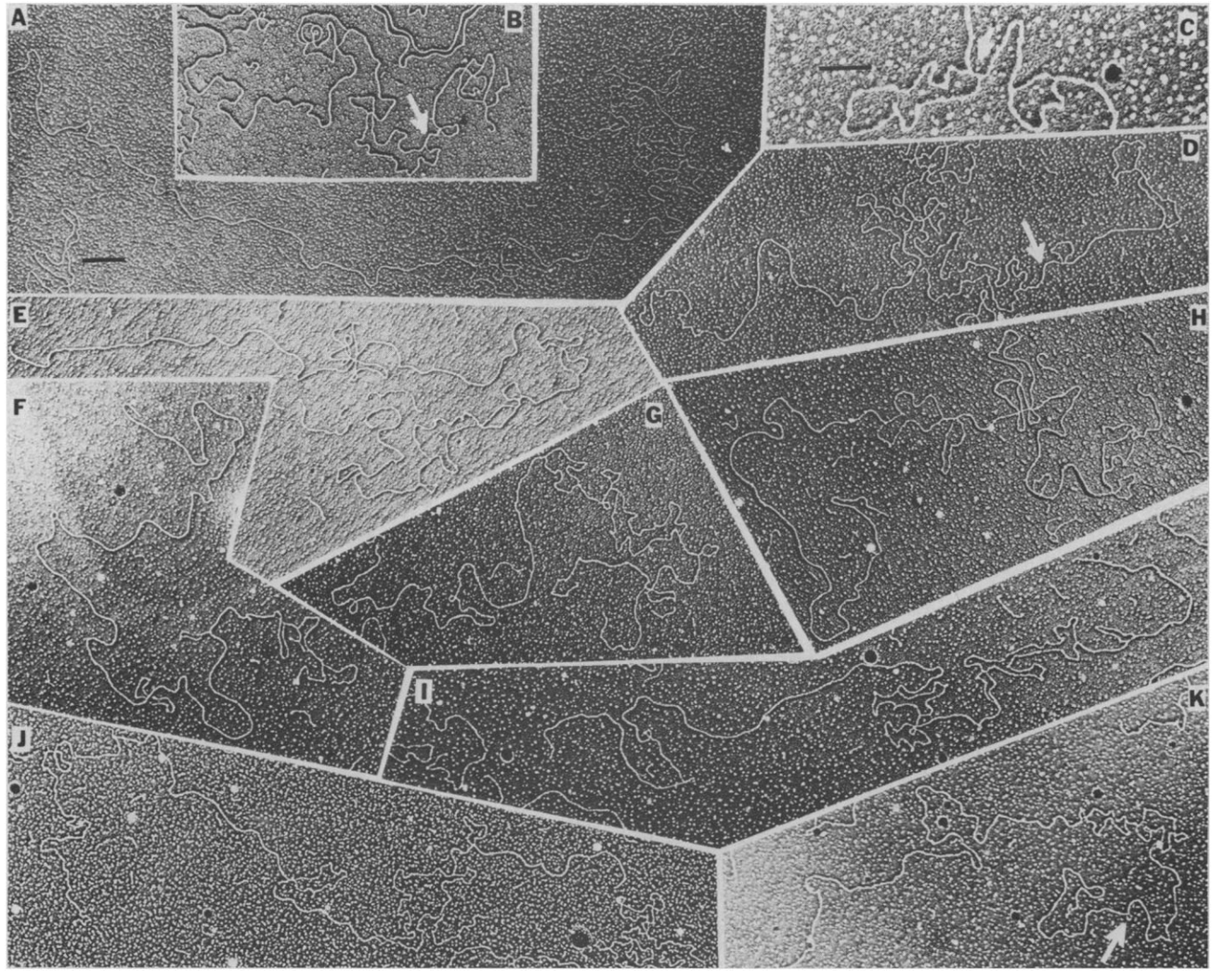


Fig. 2. Heteroduplex analysis of Charon phages. Heteroduplexes made by combining DNA strands from genetically different phages (*31*) were examined in the electron microscope. The line diagram identifies each heteroduplex and labels the loops and bifurcations where DNA strands do not pair. The calibration bar in panel (A) refers to double-stranded DNA and applies reasonably to all panels except (C). The calibration bar in panel (C) refers to single strands and applies only to that panel. The identification of phages not shown in Fig. 1 is as follows: Ch3ΔLac was made from Charon 3 by deleting its Eco RI Lac operator–containing fragment. Bio16a, panel (C), is equivalent to λ except for a small substitution of *bio* DNA to the right of *att*. 4M121 was obtained by inserting unrelated DNA from phage mu into Charon 4 (*32*). bio256nin has the same *bio* substitution and *nin*

the QSR80 substitution, has no sites to the right of *nin*5 for any of nine restriction enzymes we have tested that are potentially suitable for cloning (*11*). NRE has no visible nonhomology with λ in heteroduplexes (see Fig. 2, i and j), suggesting that the contribution of ϕ80 is slight.

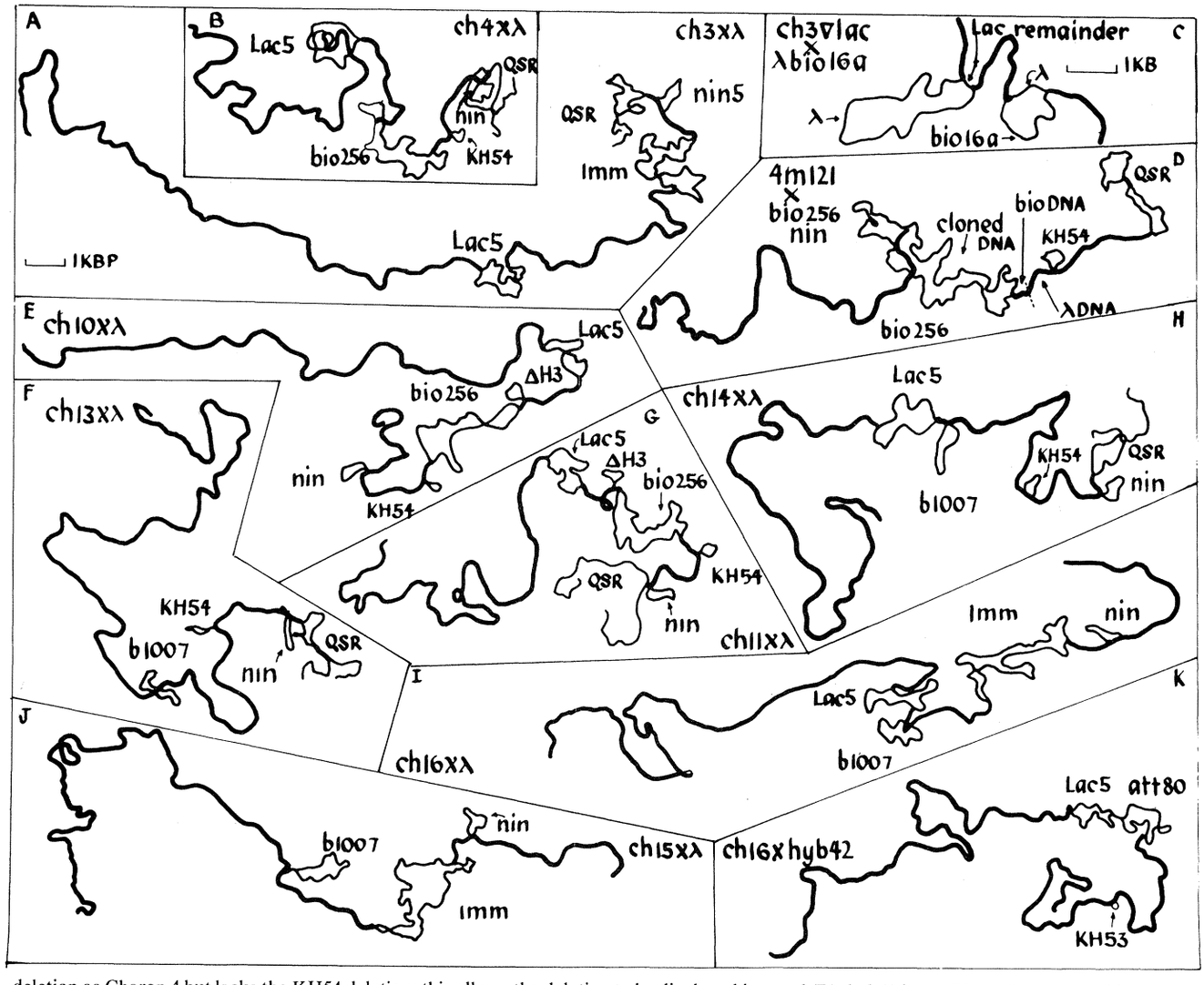
Some of the Charon phages have had amber mutations, *A*am32 and *B*aml (*12*), introduced in their capsid genes in order to enhance biological containment. These phages are designated with an A (for example, Charon 3A is derived from Charon 3 by incorporation of these mutations).

Verification of vector structure. It is most important to verify the structure of each vector. The best way to determine whether deletions and substitutions are present and to detect unexpected structural changes is by heteroduplex mapping (*31*). All 16 vectors were analyzed by this technique. The forms observed in the electron micrographs were completely consistent with the structures of the phages shown in Fig. 1. Electron micrographs of the heteroduplexes between DNA from wild-type λ and from eight of the vectors are shown in Fig. 2, together with three other informative heteroduplexes.

Heteroduplex analysis has also proved valuable for determining the amount of bacterial DNA remaining in some of the vectors after they have been used for cloning with Eco RI. An example of this is shown in Fig. 2c, where the bacterial DNA remaining from *lac*5 after the dispensable portion of Charon 3 had been excised with Eco RI is displayed directly as a single strand; it is less than 200 base pairs in length. The bacterial DNA remaining after Eco RI cloning with vectors that contain *bio*256 was similarly determined by subtracting the duplex lengths indicated by arrows in Fig. 2, d and b; this measured 980 ± 40 base pairs.

Genetic characteristics of all the vectors have been verified by plating on suitable indicator strains. Characteristics examined included the immunity type, the presence of the *lac*5 and *bio*256 substitutions, and the presence of amber mutations (*13*).

Each vector DNA was also digested with restriction enzymes, and the sizes of DNA fragments were determined on agarose gels. By analyzing these, and other related data, the positions of the restriction sites indicated in Fig. 1 were determined. The Sst I sites of Charon phages 1, 2, 5, 6, 7, 8, 9, 10, and 12 have not yet been determined.



deletion as Charon 4 but lacks the KH54 deletion; this allows the deletion to be displayed in panel (D). hyb42 is λ*att*80*imm*80*nin*; its wild-type ϕ80 immunity region permitted display of the KH53 deletion. The arrows in the photograph panels (D) and (B) indicate duplex segments next to the KH54 marker; subtraction of their lengths provided a measurement of *E. coli* DNA remaining in Charon 4 clones; this residual *bio* DNA is indicated in the tracing of panel (D). The arrow in the photograph panel (C) indicates *E. coli* DNA remaining in Charon 3 clones. The arrow in photograph panel (D) shows the KH53 deletion.

Vectors for cloning a variety of sizes of DNA fragments. Since λ capsids cannot accommodate DNA molecules outside the size range 38 to 53 kilobase pairs (kbp) (*4, 10, 14, 34*), each λ phage vector has a maximum and some have a minimum size of DNA fragment that can be cloned with a given restriction enzyme. The size limits for each of the Charon phages are listed in Table 1. These ranges depend not only on the positions of restriction cuts but also on the sizes of deletions and substitutions in the essential region of the vector. Any decrease in the size of the vector genome results in a corresponding increase in the maximum fragment that can be cloned, and may result in a greater minimum size. Charons 1, 4, 8, 9, 10, 11, 13, and 14 are all suitable for cloning large fragments. Charons 8 and 9 have the largest capacities, and approach the theoretical maximum for a nondefective λ vector. Among the EK2-certified Charon phages, Charon 4A can be used for cloning large Eco RI fragments.

Vectors for cloning with more than one restriction enzyme. When small DNA fragments are to be cloned, a single restriction site in a nonessential portion of the phage DNA is sufficient. Several of the Charon vectors can be used with more than one restriction enzyme in this way (Table 1). If only one site occurs for each enzyme, the heterogeneously terminated fragments obtained by digestion with all possible pairs of the enzymes can be cloned. Heterogeneously terminated vectors and fragments prevent self-cyclization reactions. The EK2-certified phage Charon 16A can be used in this way with Eco RI and Sst I.

For cloning large fragments with multiple enzymes, a "magic" arrangement would be to have a series of different restriction sites arranged in the same order at each end of the dispensable region. With such an arrangement, restriction enzymes could be used either singly or in any possible paired combinations to clone large fragments. Charon 9 is the closest approximation to such a vector in our series.

Vector functions indicating successful cloning. A powerful indicator of successful cloning is available in Charon phages 1, 4, 8, 9, 10, 11, 13, and 14, which all have a lower limit to the size of cloned DNA they can accept (see Table 1). In these phages, when the dispensable portion of

Table 1. Characteristics of Charon cloning phages. The DNA cloning vectors and their genotypes are presented. The individual mutations are discussed in the text and in (*6*). Typical growth titers obtained by the PDS method are given, and they do not differ significantly with either the A series or the clone-bearing derivatives of the vectors. The total DNA length of each vector is given as a percentage of wild-type λ, determined from buoyant densities. These vectors can be used with various restriction enzymes. For each of the useful vector-enzyme combinations, the left- and right-end fragment lengths are given as percentage of λ, as determined by agarose gel electrophoresis or electron microscopy (or both). The corresponding cloning capacity is given in kilobase pairs, assuming that the packaging limits on the genome are from 38 to 53 kbp. The symbols p_L and p_{Lac}, under the heading "promoter control," indicate whether or not these major vector promoters would be expected to transcribe the cloned fragment. Other promoters (p_R', p_{re}, or p_{rm}) might also be useful in some cases. Suggested genetic tests to indicate successful cloning are: (a) colorless plaques on Lac$^-$ cells provide definite indication of foreign DNA insertion; (b) colorless plaques on Lac$^+$ cells provide definite indication of foreign DNA insertion without reinsertion of dispensable vector fragment; (c) clear plaques provide definite indication of foreign DNA insertion; (d) combination of colorless plaques in Lac$^+$ cells with Bio$^-$ phenotype provides definite indication of foreign DNA substitution without reinsertion of either dispensable fragment. Bio$^+$ phenotype is detected by a ring of bacterial growth around plaques on Bio$^-$ cells on biotin-deficient plates; (e) lack of ability to plate on *pol*A$^-$ cells provides definite indication of foreign DNA insertion; (f) colorless plaques on Lac$^+$ cells indicate removal of dispensable fragment but does not ensure that insertion of foreign DNA has taken place; (g) plaque formation indicates DNA insertion but does not ensure that reincorporation of dispensable fragments has not taken place; and (h) no particularly useful tests currently available. Not all genetic tests have been verified in all cases.

Vector	λ genotype	Typical growth	Total DNA length (%λ)	Fragment lengths in percent λ			Cloning capacity (kbp)	Promoter control	Genetic tests
					Left end	Right end			
Charon 1	*lac*5 KH100 BW1 *nin*5 *QSR*80	3×10^{10}	100.5	Eco RI:	39.5	20.6	9.1–23.2		(g)
Charon 2	*lac*5 *att*80 *imm*80 KH53 BW2 *nin*5 *QSR*80	2×10^{10}	95.1	Eco RI:	39.5	55.6	0–5.8	p_{Lac}	(a)
Charon 3	*lac*5 *imm*80 KH53 BW2 *nin*5 *QSR*80	6×10^{10}	98.7	Eco RI:	39.5	42.6	0–12.3	p_L	(f)
Charon 4	*lac*5 *bio*256 KH54 BW1 *nin*5 *QSR*80	1×10^{10}	93.6	Eco RI:	39.5	22.5	8.2–22.2	p_L	(d)
Charon 5	DUPL b189 *bio*1 KH100 BW1 *nin*5 *QSR*80	2×10^{10}	93.5	Eco RI:	72.7	20.8	0–7.1		(h)
Charon 6	DUPL b189 *bio*1 *imm*434 BW1 *nin*5 *QSR*80	1×10^{10}	88.6	Eco RI:	68.3	19.9	0–9.2		(c)
Charon 7	DUPL b189 *bio*1 *imm*434 BW1 *nin*5 NRE	2×10^{10}	86.2	Eco RI:	68.3	17.6	0–9.9		(c)
				Hind III:	67.3	18.6	0–9.9		(c)
				Eco RI/Hind III:	67.3	17.6	0–10.4		(c)
Charon 8	*lac*5 *imm*434 BW1 *nin*5 NRE	5×10^{10}	92.8	Eco RI:	39.5	17.6	10.6–24.6		(g)
				Hind III:	48.5	18.5	5.7–19.8		(g)
Charon 9	*lac*5 KH100 BW1 *nin*5 NRE	2×10^{11}	97.6	Eco RI:	39.5	18.3	10.2–24.3		(g)
				Hind III:	48.5	17.6	6.2–20.2		(g)
				Eco RI/Hind III:	39.5	17.6	10.6–24.6		(g)
				Sst:	42.0		≥ 13.2	p_L	(h)
				Sst/Hind III:	42.0	17.6	9.3–23.4		(g)
Charon 10	*lac*5 ΔH3 *bio*256 KH54 BW1 *nin*5 NRE	3×10^{10}	85.3	Eco RI:	39.5	20.2	9.2–23.4	p_L	(b)
				Hind III:	52.2	33.1	0–10.7	p_L	(h)
				Sst:	42.0	34.0	1.3–15.3	p_L	(b)
				Hind III/Sst:	42.0	33.1	1.7–15.7	p_L	(b)
Charon 11	*lac* 5 ΔH3 *bio* 256 KH54 BW1 *nin* 5 *QSR*80	5×10^{10}	89.0	Eco RI:	39.5	22.5	8.2–22.2	p_L	(b)
Charon 12	DUPL b189 KH54 BW1 *nin*5 *QSR*80	1×10^{11}	91.0	Eco RI:	64.2	26.8	0–7.9	p_L	(e)
Charon 13	b1007 KH54 BW1 *nin*5 *QSR*80	1×10^{11}	83.5	Eco RI:	44.5	26.8	2.4–16.7	p_L	(g)
Charon 14	*lac*5 b1007 KH54 BW1 *nin*5 *QSR*80	2×10^{10}	83.7	Eco RI:	39.5	26.8	5.0–19.1	p_L	(b)
Charon 15	b1007 *imm*80 KH53 BW2 *nin*5 NRE	1×10^{11}	86.0	Eco RI:	44.5	41.5	0–10.4	p_L	(h)
				Hind III:	48.5	37.5	0–10.4	p_L	(h)
				Eco RI/Hind III:	44.5	37.5	0–12.3	p_L	(h)
Charon 16	*lac*5 b1007 *imm*80 KH53 BW2 *nin*5 NRE	3×10^{10}	86.0	Eco RI:	39.5	46.5	0–10.4	p_{Lac}	(a)
				Sst:	42.0	44.0	0–10.4	p_{Lac}	(a)
				Eco RI/Sst:	39.5	44.0	0–11.6	p_{Lac}	(f)

DNA has been removed, the resulting vector DNA falls below 38 kbp and therefore is too small to be packaged. Only when an additional piece of DNA is inserted into the vector will a viable phage be produced (*4*). The ability to form a plaque is, therefore, a strong indicator of successful cloning. Reinsertion of a fragment or fragments from the dispensable portion will, of course, also yield viable phage. Since it is difficult to remove these dispensable fragments physically, it is advantageous to be able to detect their reinsertion genetically. It is also desirable to reduce the number and kinds of fragments from the dispensable region, so as to reduce the number of genetic tests and to reduce competition during cloning between the undesirable reinsertion fragments and the desired foreign DNA fragments. Charons 10 and 11 have a single dispensable Eco RI fragment that can be detected, when inserted in either orientation, either by plaque color on XG (defined below) plates or by tests for *bio* function.

For cloning small fragments, vectors must be used that do not have a minimum size limit. Indicators of successful cloning in these vectors can be obtained by arranging for the cloning site to be within a nonessential gene whose function can be monitored. Insertional inactivation of the nonessential gene by the cloned fragment will then indicate success. Three such indicator systems are potentially available for Charon phages. Insertion into Charon phages 6 and 7 should make the plaques clear. Insertion into the *red* gene of Charon 12 should make the phage unable to plate on *polA*$^-$ cells. Insertion into the *lac*z gene of Charons 2 or 16 should render the plaques colorless on Lac$^-$ cells on XG plates.

Use of lac5 *as an indicator function*. As was discussed above, several of the Charon vectors, including all three EK2-certified phages, contain the *lac*5 substitution, which carries the *E. coli* gene for β-galactosidase (*lac*z). When the chromogenic substrate 5-chloro-4-bromo-3-indolyl-β-D-galactoside (XG) is included in the plating medium, phage carrying *lac*5 give vivid blue (indigo) plaques. In order to use this reaction for indication of potential success in cloning, it was necessary to gain some understanding of the mechanism of formation of blue plaques. In *lac*5 the promoter, operator, and *z* gene are oriented leftward, and an Eco RI site is located within the *z* gene (*15*). This cut is within 200 base pairs of the left end of the substitution. To determine the effect of an interrupted *lac*z gene on plaque color, phages were constructed from Charon 3 and Charon 11 in which the Lac region had been cut out by Eco RI, turned around, and reinserted so that its orientation (see below) was changed from "n" to "u," and the *z* gene was interrupted. These phages, the parents, and other containing insertions of unrelated DNA were tested on Lac$^+$ and Lac$^-$ indicator bacteria on plates containing XG (80 μg/ml). We found that the development of blue plaques depended on the *lac* genotype of the plating bacteria as well as that of the phage. Dark blue plaques were always produced when the phage contained *lac*5 in the normal arrangement, and the plaques were always colorless when the fragment containing *lac*5 was removed. However, the phages with an interrupted *z* gene gave pale blue plaques on Lac$^+$ cells but colorless plaques when the indicator bacteria were Lac$^-$ (see cover). We conclude that a blue plaque can be produced either directly by expression of a β-galactosidase gene supplied by the infecting phage or indirectly when the many copies of the Lac operator DNA produced during phage growth bind the Lac repressor available in the cell, causing derepression of the bacterial *lac* operon.

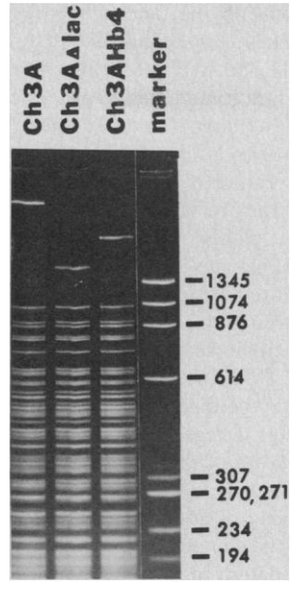


Fig. 3. Detection of inserted DNA in Charon 3A by Hha I digestion. Charon 3AHb4 was made by replacing the Eco RI Lac operator–containing fragment of Charon 3A with mouse globin cDNA. Charon 3AΔLac is Charon 3A without the Eco RI fragment. DNA from each of these phages was digested with Hha I and compared by 6 percent polyacrylamide gel electrophoresis. A Hae III digest of φX174 DNA was used as a calibration mixture. The marker sizes are indicated in base pairs. The largest fragment (> 1345 base pairs) is the piece of interest. If it is larger than that in Charon 3AΔLac, then an insert is present (as in Charon 3AHb4). If it is replaced or accompanied by new smaller pieces, then an insert with an Hha I site (or sites) is present (no example of this is shown).

To summarize, if we use as examples Eco RI cloning with the EK2-certified vectors, potential success with Charon 16A is indicated by loss of the Lac function detected by colorless plaques on XG plates with Lac$^-$ bacteria. Potential success with Charon 4A is indicated by obtaining viable phages giving colorless plaques on XG plates with Lac$^+$ bacteria. [A separate test is required for the absence of the Bio fragment (*13*).] Potential success with Charon 3A is indicated by colorless plaques on XG plates with Lac$^+$ bacteria. Since Charon 3A phages that have lost the Lac fragment without insertion of foreign DNA cannot be distinguished genetically from a clone, additional tests are required, such as hybridization to a suitable probe or gel electrophoresis of a restriction enzyme digest of the phage DNA (Fig. 3).

Controlled transcription of cloned fragment. The cloning sites in many of the Charon phages can have their transcription controlled by the phage leftward promoter (p_L) or the Lac promoter. This property has already proved valuable in mapping promoters in *E. coli* fragments specifying ribosomal proteins that were cloned in Charon phages 3 and 4 (*16*).

Cloning with Charon phages. The procedure that we have developed for constructing clones differs significantly from those previously described; the conditions for ligation of fragments have been chosen to favor assimilation of all target fragments into giant concatenates rather than to attempt to limit the reaction at the dimer stage. The vector phage DNA is annealed to join its ends. (Lambdoid phages have mutually complementary single-stranded sequences on the ends of their DNA molecules.) The vector and target DNA's are digested with the desired restriction enzyme, which is then inactivated with diethyl pyrocarbonate (*17*). In order to generate recombinant DNA molecules, the vector and target DNA's are mixed. Their relative proportions are adjusted so that the molar concentration of target DNA fragments does not exceed that of the vector DNA, and the total DNA concentration is kept high (*18*) to ensure that the formation of recombinant molecules competes effectively with monomolecular cyclization reactions, particularly of short target fragments. The mixture is incubated with T4 ligase to join the molecules covalently. Viable phages can then be obtained by transfection of *E. coli* spheroplasts (*19*) or calcium-treated *E. coli* (*20*), or by in vitro encapsidation into λ capsids (*21*). We presume that λ-sized chimeric molecules are either extracted directly from concatenates by the λ packaging function, or

that circles are first generated by recombination within the transfected *E. coli* cell.

Vectors with a high yield. An important safety feature for any cloning vector is its ability to be grown in high yield, so that only small volumes of culture are required for production purposes. The Charon phages and clones derived from them can usually be propagated to at least 10^{10} phage per milliliter by our PDS (preadsorb-dilute-shake) method (*22*). After lysis, 3 ml of chloroform is added; this immediately kills all surviving bacteria without affecting the phage. Typical yields for some of the vectors are shown in Table 1. Yields of phages containing cloned fragments have usually been in the same range.

Recovery of cloned DNA. Two convenient methods have been devised for recovering cloned DNA fragments from Charon phages. The first method is general and applies to any clones containing a single restriction enzyme fragment smaller than about 2 kbp. In this method the clone-bearing phage DNA is prepared, heated at 55°C in 2*M* NaCl to anneal the cohesive ends, dialyzed, cut with the restriction enzyme, and centrifuged for 4 hours at 40,000 rev/min (SW50.1 rotor) at 15°C. The small cloned fragment remains in the supernatant and can be recovered free of the large vector fragment by ethanol precipitation.

The second method is useful in selected cases, and we have used it for studying mouse globin complementary DNA (cDNA) clones that were obtained by procedures similar to those that have been used with plasmids (*23*). The purification method is based on the fact that the largest DNA fragment in Hha I restriction enzyme digests of Charon 3A ΔLac (see Fig. 2 legend) contains the single Eco RI site of this phage. Provided that the cloned piece of DNA inserted into this site does not contain an Hha I site, the size of the cloned DNA fragment can easily be determined from the size of the largest fragment in an Hha I digest. The position of this fragment after polyacrylamide gel electrophoresis is convenient for preparative purposes. Figure 3 illustrates this point with one of our mouse globin cDNA clones.

Hybrid phage nomenclature. Charon clones are named by appending to the name of the vector one or more letters denoting the source of inserted DNA, followed by an isolation number. For example, Charon 3AHb4 was our fourth isolation candidate for a mouse globin clone. The orientation of an inserted fragment can be denoted "n" (natural) or "u" (unnatural), depending on whether the coordinate system assigned to the target fragment increases from left to right in agreement with λ's. In Charon 11, the Lac and Bio DNA's are in the "n" orientation since the clockwise "time-increasing" direction of the *E. coli* map corresponds to the left-right direction in λ.

Biological containment. Guidelines have been set up by the National Institutes of Health for assessing the suitability of host-vector systems for the cloning of DNA from different sources (*2*). When DNA from higher organisms, such as mammals, is being cloned, a host-vector system with a biological rating of EK2 is required.

The NIH guidelines specify (*2*) that: "For EK2 host-vector systems in which the vector is a phage, no more than one in 10^8 phage particles should be able to perpetuate itself and/or a cloned DNA fragment under nonpermissive conditions designed to represent the natural environment either (a) as a prophage or plasmid in the laboratory host used for phage propagation or (b) by surviving in natural environments and transferring itself and/or a cloned DNA fragment to a host (or its resident lambdoid prophage) with properties common to those in the natural environment." Charon phages 3A, 4A, and 16A in conjunction with host bacteria DP50 (χ1953), or DP50SupF (χ2098) have now been certified as meeting these criteria by the director of NIH following the recommendation of his Recombinant DNA Advisory Committee.

The accumulation of data on safety and the certification process itself took more than a year and involved our submitting eight documents detailing experimental results concerning safety (*24*). A phage working group, appointed by the NIH Recombinant DNA Advisory Committee and headed by W. Szybalski, met three times to analyze the results. They made several useful suggestions to us for additional experiments prior to recommending that the systems be certified.

In the following, we present a brief synopsis of the safety features of the λ vectors, including (Table 2) the results of three safety experiments that were considered particularly germane by the phage working group.

General safety features of λ phages. Foreign DNA cloned in a λ phage might be propagated outside the laboratory in

Table 2. Gene transfer between Charon A phages and bacteria. Exchange of genes in both directions between Charon A phages and bacteria was estimated by considering three cases. (Experiment 1) Gene transfer from phage to productive host: Lysates of Charon A phages were prepared and the titers for phage (at lysis) and for total bacteria (after overgrowth) were determined; the λ^s*lacz*$^-$y$^+$*sup*E bacterium CSH18 was used as host. Lac$^+$ bacteria (those that had stably incorporated the *lacz* gene function from the phage) were measured in the overgrown cultures. The ratio of the stable associates after overgrowth to the phage titers at lysis were calculated. (Experiment 2) Gene transfer from phage to nonproductive host: Charon A phages were adsorbed at a 1 : 1 ratio to λ^{s}Lacz$^-$y$^+$sup^0 bacteria (M96CF′) and to two heteroimmune lysogens of this strain (φ80*imm*21 and λ*imm*21). Lac$^+$ bacteria produced by the transfer of *lacz* from phage to host were measured and expressed as a fraction of the phage input. (Experiment 3) Gene transfer from nonproductive host to phage: The experiment was similar to experiment 2 except that the phage produced by the infected cells were studied. Phages that had either of the safety features, immunity deletion or A^-B^-, replaced by genes from the host lysogens (marker rescue) were assayed. Transfer of *imm*21 to Charon 4A phage was measured by titration on a λ lysogen of the *sup*F strain Ymel; for Charons 3A and 16A, a φ80 lysogen was used. Transfer of A^+B^+ genes from lysogen to Charon phage was assayed by counting the number of blue plaques on the *sup*^{0} strain W3350 on XG plates. N.T., not tested.

Vector	Ch3A	Ch4A	Ch16A
Experiment 1. Phage to productive host			
Phage/ml*	1.0×10^{11}	1.0×10^{10}	3.0×10^{10}
Bacteria/ml†	5.8×10^{9}	4.7×10^{9}	3.1×10^{10}
Lac$^+$bacteria/ml	4.9×10^{4}	5.2×10^{4}	1.5×10^{4}
Lac$^+$bacteria/phage	4.9×10^{-7}	5.0×10^{-6}	5.0×10^{-7}
Experiment 2. Phage to nonproductive host (Lac$^+$ cells obtained per input phage)			
Nonlysogen	8.4×10^{-9}	1.8×10^{-8}	N.T.
φ80*imm*21 lysogen	7.0×10^{-7}	2.5×10^{-7}	$<2.9 \times 10^{-8}$
λ*imm*21 lysogen	4.5×10^{-6}	1.2×10^{-6}	2.7×10^{-6}
Experiment 3. Nonproductive host to phage (output phage with rescued imm21 *or* A^+B^+ *per input phage)*			
Nonlysogen	$<10^{-6}$	$<10^{-6}$	$<10^{-6}$
φ80*imm*21 lysogen:			
*imm*21			1.0×10^{-5}
A^+B^+	3.6×10^{-4}	2.0×10^{-5}	1.3×10^{-5}
λ*imm*21 lysogen:			
*imm*21	$<5.7 \times 10^{-5}$	$<3.9 \times 10^{-5}$	1.2×10^{-4}
A^+B^+	1.1×10^{-3}	2.4×10^{-3}	5.9×10^{-3}

*At harvest. †After overgrowth.

two ways: (i) lytically, if the clone-bearing phage found a suitable host in the environment and started a lytic replication cycle before it had been inactivated; or (ii) as a lysogen or plasmid, if the phage DNA containing the cloned segment became incorporated into the genome of a bacterium, or if the phage DNA became established as a defective (nonlytic) plasmid in a bacterium, and if the bacterium could then grow outside the laboratory.

In order for λ to be propagated lytically outside the laboratory, phage particles must be capable of surviving in the natural environment. We find that fewer than 1 in 10^{10} phages survive exposure to *p*H 3 for 2.75 hours (mimicking stomach conditions); fewer than 2 in 10^7 survive 30 minutes in detergent (1 percent sodium dodecyl sulfate); fewer than 2 in 10^4 survive 6 hours of drying on a laboratory bench; fewer than 3 in 10^6 survive 120 hours in raw sewage. Thus, there are initial inactivation barriers to dissemination.

In order for λ to multiply in the natural environment, the phage must repeatedly encounter sensitive bacterial hosts. Typical raw sewage contains 10^7 bacteria per milliliter. Calculations show that self-sustaining lytic replication requires at least 10^5 sensitive bacteria per milliliter, so that phages can encounter bacteria sufficiently often for replication to overcome inactivation (*25*). Yet, of more than 2000 strains of *E. coli* independently isolated from the wild or from hospital patients, none was λ sensitive (*26*). Consequently sewage is unlikely to be able to sustain a lytic replication of λ. The lack of ability of *E. coli* in the wild to replicate λ phages is borne out by our measurements of decreasing titers of λ phages on shaking with raw sewage (Fig. 4). Furthermore, λ cannot inject its DNA into bacteria below 15°C (*27*).

With the Charon phages, it has also been possible to decrease the number of potential natural hosts by including amber mutations in the phages. Such phages require a suppressor transfer RNA (tRNA) in the host bacterium of a type not usually found in wild-type *E. coli*. A further decrease in the number of potential natural hosts is obtained by propagating the phage in the laboratory on bacteria that do not modify the phage DNA; the DNA is then vulnerable to attack by the restriction endonucleases found in many wild-type *E. coli* strains.

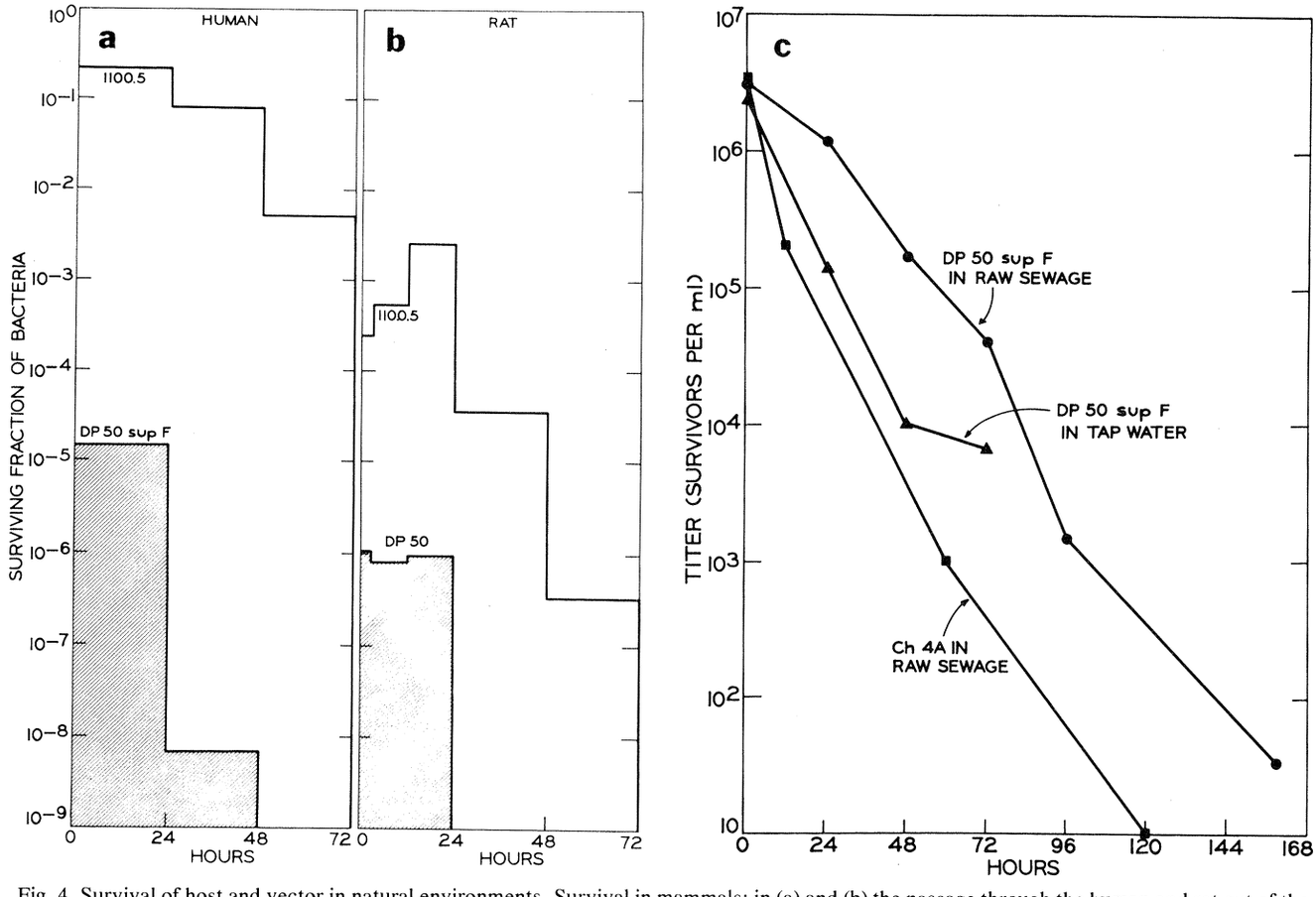


Fig. 4. Survival of host and vector in natural environments. Survival in mammals: in (a) and (b) the passage through the human and rat gut of the debilitated laboratory hosts DP50 and DP50SupF and, for comparison, a nondebilitated strain of *E. coli* K12 [1100.5 (*28*)] was examined. These strains are resistant to nalidixic acid, and titers can be determined on eosin–methylene blue–minimal galactose plates supplemented with vitamin-free Casamino acids and containing nalidixic acid (100 μg/ml). Such plates eliminate growth of native gut bacteria much better than plates made with yeast extract, and permit discrimination between Gal^+ (1100.5) and Gal^- (DP50 and DP50SupF) bacteria. (a) A mixture of 3×10^{11} DP50SupF and 9.4×10^8 1100.5 was fed in 250 ml of milk to three humans. Bar heights represent the averaged fraction of bacteria surviving gut passage during the time intervals defined by the end points of each bar. The total fraction of DP50SupF surviving is 1.4×10^{-5}, which is 4.7×10^{-4} that of 1100.5. (b) Broth (0.25 ml) containing 8×10^8 DP50 was placed on the tongues of three rats, and 1.6×10^9 1100.5 bacteria were fed to three other rats in the same manner. The total fraction of DP50 surviving is 2.9×10^{-6}, which is 8.3×10^{-4} that of 1100.5. In neither species was there any indication of colonization by any K12 strain. Survival in sewage. In (c) City of Madison raw sewage, including University of Wisconsin effluent, was collected from a pond immediately upstream of the treatment plant; it had a titer of 10^7 bacteria per milliliter measured on L plates. The inactivation of the EK2 vector, Charon 4A, shaken in raw sewage at 37°C, and of the debilitated host, DP50SupF, shaken in raw sewage and tap water at room temperature, was examined. The titer of Charon 4A was determined by counting blue plaques on XG plates; DP50SupF was determined as above. The initial first-order rate constants for inactivation in sewage were 1.0 day^{-1} (bacteria) and 5.6 day^{-1} (phage). The averages over the entire experiment were 1.7 day^{-1} (bacteria) and 2.6 day^{-1} (phage).

We conclude that, as far as dissemination by lytic processes is concerned, λ offers an inherently high degree of biological containment, which has been further increased in the Charon phages.

Escape of the phage-cloned DNA in a bacterial lysogen or plasmid must be considered from two aspects—the bacterium might be the host used for propagation in the laboratory, or it might be a naturally occurring one encountered by a clone-bearing phage after accidental release into the environment. In either case the self-replicating ability of bacteria, in contrast with the inability of phage to replicate without a host, makes dissemination of a cloned fragment by the bacterial route potentially serious. We have, therefore, focused much of our safety strategy on designing the vector so that its replication will be confined to the lytic mode.

The ability to form lysogens or plasmids has been reduced in Charon phages 3A, 4A, and 16A by deletions KH53 or KH54 in the immunity regions of the phages, by the *nin*5 deletion (coupled with wild-type N function) which enhances lytic functions, and, in the case of Charon phages 4A and 16A, by the elimination of the gene *int* and the *att* site, which are needed for efficient insertion of the phage genome into the bacterial chromosome.

The effectiveness of these strategies can be measured by determining what proportion of bacteria surviving an encounter with a Charon phage acquire the cloned fragment of DNA in some stable association. The *lac*5 *E. coli* substitution carried on Charons 3A, 4A, and 16A is a convenient model cloned segment, provided that test bacteria are used which provide the *lac*y (permease) function and do not contain the *lac*z (β-galactosidase) function. Appropriate test bacteria were constructed for this purpose (*28*).

The first experiment shown in Table 2 was designed to measure the formation of stable associates with a laboratory host during productive growth. Charon phages were grown by the PDS method on Lacz$^-$y$^+$ cells. To mimic a worse case, the lysates were not harvested at the normal time but, instead, were allowed to overgrow until surviving bacteria had reached a high titer. Chloroform was not added. Surviving cells were separated from the free phage, diluted into broth, and shaken for 90 minutes to permit stable associates to become established and to permit unstable associates to segregate. The bacteria were then plated on XG indicator plates with lactose as the sole carbon source. Reconstruction experiments were done to verify our ability to demonstrate very low levels of Lac$^+$ bacteria in lysates. The titers of phage, total bacteria, and Lac$^+$ bacteria are listed in Table 2 (experiment 1). The results show that the ratio of undesirable products (Lac$^+$ bacteria) to desirable products (phages) is of the order of 10^{-6}. (The majority of the bacteria surviving these lytic infections were shown to be λ-resistant.) When lysates were harvested at the proper time, these ratios were more favorable by a factor of 10^3, and chloroform is routinely used to kill all the bacteria in lysates. Nevertheless, the phage working group recommended using a host bacterium for the productive growth of the phage that is able to provide an additional safety factor over that provided by *E. coli* K12. This recommendation was implemented by our using DP50, as described below.

The second experiment in Table 2 was undertaken to test the likelihood that a recombinant phage that escaped into nature might subsequently be stably integrated into a naturally occurring bacterium. Although the probability of an encounter under dilute conditions with a nonpermissive bacterium that could adsorb λ would be low, as discussed above, it might be cause for concern if the integration step turned out to be highly efficient. Again worse cases were tested in which the bacteria were λ-sensitive, lacked an amber suppressor, and carried prophages having a high homology (λ *imm*21) or moderate homology (φ80 *imm*21) with the Charon phages. We also tested nonlysogenic bacteria. In these experiments, Charon phages were adsorbed efficiently to the bacteria at a ratio of about 1 : 1 and then diluted into broth as before. The efficiency of transfer of a model cloned segment to the nonpermissive lysogen relative to the number of input phage is listed in Table 2 (experiment 2). These data show that the formation of stable associations is far from being efficient.

The third experiment in Table 2, suggested by the phage working group, provides data on marker rescue. The marker rescue experiments were designed to test the possible outcome of a phage escaping as a particle, and surviving in the natural environment long enough to find a rare bacterium (to which it can adsorb) which harbors a prophage that can remove by marker rescue the phage mutations introduced for safety. Recombination between such a prophage and the clone-bearing phage could create a new phage with some of its safety features compromised. The protocol for this experiment again involved adsorbing Charon A phages to λ-sensitive lysogens lacking an amber suppressor. The release of Lac-containing phages that had lost either the amber mutations or the immunity deletions was then measured (Table 2, experiment 3).

The results show that the frequencies of loss by marker rescue of the amber mutations on the left side of the cloned segment are around 3×10^{-3} and of the immunity mutations on the right side are around 5×10^{-5}. Both of these events would not fully compromise the safety of the Charon system, and they would be but a small part of an extensive chain of improbable events. The results indicate that these probabilities are indeed worst case estimates because when the homology between the vector and the prophage was reduced, as would be the case in most strains from nature, the rescue frequencies were reduced.

Bacterial hosts. The bacterial hosts, DP50 and DP50SupF, were selected for the Charon phage system because of their additional contributions to the problem of biological containment. DP50 (also called χ1953) was constructed by Pereira and Curtiss [see (*29*)], University of Alabama. DP50SupF (also called χ2098) was constructed from DP50 by Leder *et al.* (*30*), and has the same genotype as DP50 except for the addition of *supF*58 (*su*III). The genotypes of the strains are: F^-, *dap*D8, *lac*y, Δ*gal-uvrB*, Δ*thyA*, *nal*A^r, *hsd*S, *supE*44 (plus *supF*58 in DP50SupF). The *dap*D8, Δ*thyA*, and Δ*gal-uvr*B mutations were introduced so that the bacteria themselves have a low survival in nature. This provides an extra margin of safety if the normal harvesting procedure, including chloroform treatment of the lysates, is not executed on time, and the entire culture including phage, bacteria, and a low frequency of plasmids or lysogens is inadvertantly released into the natural environment. The survival of these bacteria during shaking in raw sewage and during intestinal passage through humans and rats is shown in Fig. 4. Relative to *E. coli* K12, the survival of these strains is reduced about a thousandfold. None of the tested bacteria colonized either the rat or human gut.

The reversion rates of the bacterial mutations important for safety, *dap*D8, Δ*thy*A, and Δ*gal-uvr*B, must be considered. The *thy* and *gal* mutations are deletions and do not revert. Dap$^+$ revertants were found at about 10^{-8} in stock cultures grown from a single colony. They only accumulated to 5×10^{-8} even after 10^{12}-fold growth, so that Dap$^+$ revertants do not have any appreciable selective advantage in dap-containing medium. Moreover, none of 22 tested bacteria surviving human or rat gut passage were Dap$^+$. Thus, reversion of *dap*D8 is not likely to

compromise the safety of the bacteria to any appreciable extent. Nevertheless, as with phage, it is advisable to frequently check the genotype of the bacteria in order to avoid contamination and mistakes as well as revertants.

Since both bacteria lack the *E. coli* K modification system, only a very small proportion, about 10^{-4}, of phage propagated on them would grow on bacteria from nature having a restriction system similar to that of *E. coli* K12. Both DP50 and DP50SupF will grow the Charon A phages efficiently.

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17. The reaction mixture is made 0.1 percent diethyl pyrocarbonate (Calbiochem) by adding 0.1 volume of a freshly diluted 10 percent solution in ethanol. After incubation for 10 minutes at 37°C, other enzymes may then be added as desired.
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25. For net replication to occur, $(K_2)(B)(Y)/(K_1) > 1$, where $K_1 = 5.6\ \text{day}^{-1}$ is the initial first-order rate constant for phage inactivation by City of Madison sewage, B is the concentration of λ-sensitive bacteria in sewage, $Y = 100$ is the yield of phage per infected bacterium, and $K_2 = 7.2 \times 10^{-7}$ ml per bacterium per day (J. Salstrom, personal communication) is the second-order rate constant for phage-bacteria collision and infection.
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35. The experiments related to cloning mouse globin cDNA were done under Asilomar guidelines; we used P3 physical containment with the (now) certified EK2 vector Charon 3A. We thank many friends who have given us samples of the phages and bacterial strains, J. Nulter for the cooperation of NIAID, and W. Szybalski and W. F. Dove for critical reading of the manuscript. The name Charon phages was suggested by Eric C. Rosenvold. We thank C. Morita, D. Stephenson and V. Farkas for technical assistance; J. Richards, S. Nitz, and A. Johnson provided help with illustrations. Supported by NIH contract AI 62506, and grants GM 21812 and GM 20069. This is Paper No. 2125 from the Laboratory of Genetics, University of Wisconsin, Madison.

7 February 1977