Table 2. Infectivity of DNA. The source of DNA is abbreviated as described in the legend to Fig. 2A. In the case of pCLS4, we mean covalently closed rings. The pCLS4/Sal I refers to this DNA treated with Sal I to produce a mixture of two linear molecules: the plasmid vehicle, p, and the cloned segment CLS4. In the case of lines 6 and 7 these linear segments were religated with T4 DNA ligase (gift of Dr. F. Asubel) to produce 30 percent and 60 percent rings, some of which may be nicked. These were assayed by scanning gels stained with ethidium bromide. Leaves of *Brassica* plants (*Brassica campestris*, "Tendergreen mustard" c.v. Burpee's seed) were washed with sterile 0.15*M* NaCl containing 0.015*M* sodium citrate, *p*H 7.0 (SSC), and then treated with DNA in SSC. Two young leaves were inoculated with carborundum on each plant, each with about half the amount of DNA shown. After 1 hour, the treated leaves were washed with sterile distilled water. The affected plants show an altered pattern of pigmentation and other symptoms that generally allow unambiguous scoring after 1 month (*7*).

DNA source	Amount (μg)	Plants infected/ inoculated
1. CLMV	10.0	6/9
	0.1	4/9
2. CLMV/Sal I	3.0	0/3
	1.0	0/6
3. pCLS4	10.0	0/6
	1.0	0/6
4. pCLS4/Sal I	10.0	0/3
	1.0	0/3
5. pML21	20.0	0/2
	10.0	0/4
6. CLS4/Sal I/religated (30 percent rings)	10.0	0/6
	1.0	0/6
7. CLMV/Sal I/religated (60 percent rings)	10	0/4
	1	0/9

taining ampicillin (30μg/ml) (Wyeth) were prepared. Many ampicillin resistant colonies were selected and tested on plates containing tetracycline (25 μg/ml) (Lederle). In this way, 13 amp^R tet^S clones which were derived from five separate tubes were selected, and therefore must contain at least five independent transformants. Plasmid DNA was prepared from all 13 strains by the Brij-lysis procedure (*9*); gel analysis before and after cleavage by Sal showed that each plasmid contained a long insert that proved to be the same length as CLMV/Sal. Further digestion by Bam and Eco RI gave identical patterns. Two strains, pCLS4 and pCLS8, were selected and plasmid DNA was purified by phenol extraction, ethanol precipitation, ethidium bromide–CsCl banding, and dialysis.

We attempted to clone CLMV/Bam by an equivalent procedure involving pGM 439 (*8*). However, the plasmid DNA's showed that none contained inserts characteristic of intact CLMV.

The plasmid DNA's pCLS4 and pCLS8 were each cleaved by Sal and subjected to electrophoresis in agarose tubes (1 cm in diameter); the zone containing the inserted viral segment, now called CL, was dissolved in saturated KI and the DNA was recovered on hydroxyapatite. These DNA's were cut by Eco RI, Bam, Hind, and Hae endonucleases. The results (Fig. 2 and Table 1) indicate that CLS4 and CLS8 have identical restriction segments and that these are nearly identical, with the same restriction segments formed from CLMV/Sal. Some possible differences are marked by the asterisks in Table 1.

Infectivity studies on "Tendergreen" mustard plants were performed as described (Table 2). In each case, CLMV DNA itself displayed infectivity. In contrast, CLMV DNA that was cleaved by Sal showed no infectivity even after religation. Likewise, the clonally amplified CLS4 showed no infectivity even after religation. Although the reason for this lack of infectivity is not known, some possibilities might be mentioned. (i) Perhaps there are closely spaced Sal sites, and the small segment between them is lost. (ii) Perhaps CLMV, like certain other plant viruses, has a bipartite genome, in which case two virus particles would be required to establish infection. Perhaps the minor bands mentioned above are derived from some helper virus. (iii) Perhaps the native CLMV DNA has some crucial secondary structure that is lost on cleavage or state of modification that is not present in the cloned material.

Passage experiments were initiated in leaf homogenates in an effort to determine whether the pCLS4 was replicated in the plant or acquired the ability to cause symptoms. Again no special symptoms could be observed, even after three and four passages.

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The Effects of *Escherichia coli* and Yeast DNA Insertions on the Growth of Lambda Bacteriophage

Abstract. *The effects of Eco RI endonuclease-cleaved* Escherichia coli *and yeast (*Saccharomyces cerevisiae*) DNA fragments on the propagation of the lambda bacteriophage vectors containing them were determined on a nonmutated and a PolA* E. coli *K12 host. Observable alterations in the growth of hybrids containing yeast DNA insertions were less frequent and less extreme than those seen in hybrids containing* E. coli *DNA. A lambda–*E. coli *hybrid was selected after extensive growth on the PolA (deficient in polymerase I) host which also grew very well on the PolA$^+$ host and may have resulted from some alteration in the hybrid. Hybrids selected on the PolA host gave no evidence for the expression of polymerase I activity. No lambda-yeast hybrid made from the λgt vector lacking lambda-specific recombination (*red$^-$*) had a yield of viable bacteriophage on infection greater than two-thirds that of "wild-type" lambda.*

Recent work has focused on the preparation in vitro of viable recombinant DNA molecules containing eukaryotic or prokaryotic DNA joined to a self-replicating prokaryotic vector DNA. An important concern in these experiments is the effect of the inserted DNA on the propagation of the vector in which it is inserted. A detrimental effect is of interest from the experimental standpoint because this may lead to difficulty in preparation, complete loss of the hybrid, or selection of a sequence alteration in the hybrid DNA which allows more efficient propagation. Any effect on growth of the hybrid is of importance since it might be indicative of expression of some function by the inserted DNA in the bacterial cell.

Related to this is the possible increase in the biohazard potential if the foreign DNA enables the hybrid to propagate more efficiently than the "wild-type" vector either under laboratory conditions or in the natural environment.

These phenomena have been investigated by using either yeast (*Saccharomyces cerevisiae*) DNA (*1*) or *Escherichia coli* DNA inserted into a suitable strain of bacteriophage λ, λgt-λB (*2*). These hybrid DNA's were constructed by first cleaving the DNA's with Eco RI endonuclease and covalently joining the fragments with *E. coli* DNA ligase. Bacteriophage from 10^4 to 10^5 independent plaques obtained after calcium transfection of these hybrid DNA's into *E. coli* cells were pooled to form stocks of λ hybrids which contain most sequences of the yeast or *E. coli* genomes in the form of cloned Eco RI DNA fragments. The formation of these hybrids and the genetic selection of a specific λ–*E. coli* hybrid is described in (*2*). Similar pools of λ-yeast hybrids were used for the genetic selection of a yeast Eco RI DNA fragment expressing a specific function in *E. coli* by Struhl *et al.* (*3*).

The constitution of the original λ-yeast hybrid pool may be examined by observation of its Eco RI restriction spectra (pattern of DNA bands produced on agarose gel electrophoresis of restriction endonuclease cleaved DNA). The DNA was prepared from the hybrid pool after passing the pool through only a few generations of growth in *E. coli*; the DNA was cleaved with Eco RI endonuclease, and the fragments electrophoresed on agarose gels. The resulting Eco RI restriction spectra is shown in Fig. 1c and is compared to whole yeast DNA in Fig. 1b. They are quite similar band for band, showing similar fragment sizes and abundances, except for the three strong bands originating from the original cloning vector, λgt-λB (two at the top and one in the middle of Fig. 1c). This suggests that the yeast fragments do not generally have an extreme effect on the growth of the λ hybrids in *E. coli*. Such is not the case when hybrids formed with *E. coli* DNA are examined. Previously (*2*), it was shown that the Eco RI restriction spectra of DNA's made from λ–*E. coli* hybrid pools consist of several prominent bands unlike that of *E. coli* DNA which has no very prominent bands. These results are reproduced in Fig. 2. The conclusion from this observation is that many *E. coli* DNA fragments have a strong differential effect on the propagation of λ hybrids, in which they are present. These conclusions were confirmed by measuring the burst sizes (mean yield of bacteriophage per infected cell) of several λ–*E. coli* and λ-yeast hybrids. Specific λ–*E. coli* hybrids were often found with burst sizes either considerably larger or smaller than the original λgt-λB bacteriophage. λ-Yeast hybrids did not have widely varying burst sizes. Thus, the expression of any functions affecting the propagation of λ hybrids is less frequently observed with inserted yeast DNA than with the homologous DNA.

It is desirable to define the maximum growth potential of yeast or *E. coli* hybrids in various hosts and to isolate such efficiently growing hybrids from the pools. This was accomplished by growing the hybrid pools on a particular bacterial host for many successive generations and preparing DNA from the resulting pools for restriction spectra analysis at different stages. This approach is termed a "grow-out" experiment. The λ hybrid pools were grown on plates, about 10^6 bacteriophages being used per plate. The resulting confluent lysis (*4*) represents three or four burst cycles per plating. The *E. coli* strain used in this grow-out experiment, W3110, is a representative of "wild-type" K12 *E. coli* (*5*). Another strain, *E. coli* C600 (*5*), was used as an example of a heavily mutagenized host and gave results identical to W3110. Grow-out experiments with the λ–*E. coli* hybrid pool on W3110 are illustrated in Fig. 3, b to d. Hybrids carrying one Eco RI DNA fragment are selected by eight platings. Grow-out experiments performed with pools of λ-yeast hybrids on W3110 gave results similar to those obtained with the λ–*E. coli* hybrids, with the exception that selection did not occur quite so rapidly. After eight consecutive platings the pool consisted primarily of hybrids containing one yeast Eco RI DNA fragment as seen by the prominent band in the restriction spectrum of Fig. 1d.

The most prevalent DNA fragment present in the λ–*E. coli* and λ-yeast hybrids following the grow-out on W3110 was found in either orientation relative to the vector. This was determined by electron microscopic observations of self-renatured DNA's. The fact that different hybrids were selected with the same fragment in both orientations suggests that it is some intrinsic property of the fragment itself which is responsible for the growth properties of the hybrids containing it.

The grow-out experiments with the λ hybrids in W3110 or C600 are considered

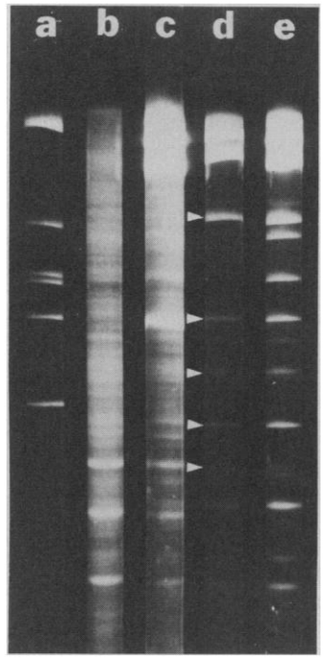

Fig. 1. Agarose gel (0.7 percent) electrophoresis of Eco RI endonuclease digests of DNA from (a) λ*c*I857, 0.2 μg, (b) linear yeast DNA, 1 μg, (c) pool of λgt-yeast hybrids made with strain 231711-136, 4 μg, (d) pool grown out on W3110, eight platings, 0.4 μg, and (e) pool grown out on H560, two platings, 1 μg. The DNA preparation, digestions, and electrophoresis were done as in (*4*). The small arrows point to fragments determined to be yeast DNA, as discussed in the text.

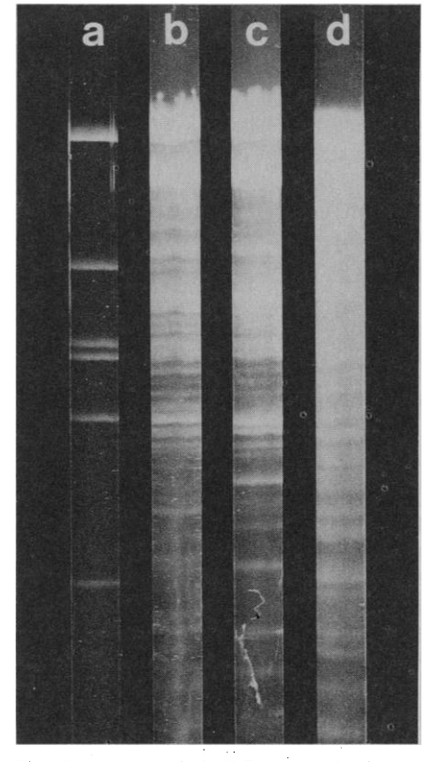

Fig. 2. Agarose gel (0.7 percent) electrophoresis of Eco RI endonuclease digests of DNA from (a) λ*c*I857, 0.2 μg, (b) pool of 400 λgt–*E. coli* hybrid plaques, 2 μg, (c) pool of 8000 λgt–*E. coli* hybrid plaques, 2 μg, and (d) *E. coli*, 3 μg [from (*2*)].

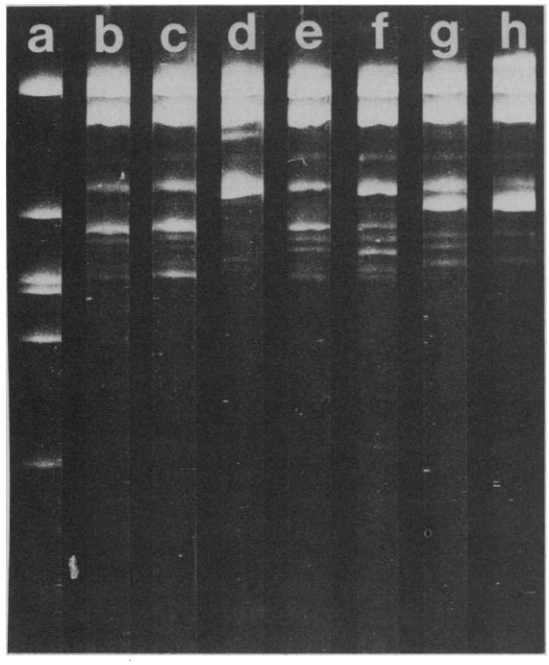

Fig. 3. Agarose gel (0.7 percent) electrophoresis of Eco RI endonuclease digests of DNA from (a) λ*c*I857, 0.2 μg, λgt-*E. coli* hybrids grown out on W3110, three (b), six (c), and eight (d) platings, 0.5 μg, and λgt-*E. coli* hybrids grown out on H560, one (e), two (f), four (g), and seven (h) platings, 0.5 μg.

to be under conditions of a nonstringent environment. A strain which provides a more stringent environment is *E. coli* H560, a *thy*$^-$ and PolAl (amber mutation in the gene for DNA polymerase I) derivative of W3110. Lambda with a nonfunctional exonuclease gene (*red*$^-$), like the initial cloning vector λgt-λB, will not form plaques on PolAl strains (*2, 6*). It was anticipated that growth of the λ–*E. coli* hybrid pool on H560 would select for those hybrids carrying the gene coding for DNA polymerase I. The pool grown on H560 for three to four generations was still heterogeneous, as shown by the Eco RI restriction spectrum of Fig. 3e. Continued growth on H560 resulted first in the enrichment for a hybrid containing one Eco RI DNA fragment and then, finally, enrichment for another hybrid containing a different Eco RI DNA fragment, as shown in Fig. 3, f to h. This fragment was present in only one orientation unlike the fragments in the hybrids selected on W3110. None of the selected hybrids carried a gene expressing DNA polymerase I activity as determined by enzyme assays of infected cell extracts (*7*) and by the lack of trans complementation with another coinfecting *red*$^-$ bacteriophage. Struhl (*8*) was able to isolate the genes analogous to *E. coli* PolA from *Klebsiella aerogenes* and *K. pneumoniae* using these same techniques.

Growth of the yeast-hybrid pool on H560 for two platings resulted in a pool that contained many different hybrids as shown in Fig. 1e. Individual isolates from these selected hybrids as well as those obtained by further growth on H560 all show moderate growth efficiency on W3110 and H560. Some of the hybrid DNA's contain the same Eco RI DNA fragments in different orientations. No evidence was found for the expression of a diffusible product resembling either DNA polymerase I or λ exonuclease. It was determined that the isolated yeast hybrids in fact contained yeast DNA by hybridizing ^{32}P-labeled complementary RNA (cRNA) from the hybrid DNA to yeast DNA from other sources. The hybridizations were conducted by first electrophoretically separating Eco RI yeast DNA fragments on agarose gels, then denaturing and eluting the DNA onto nitrocellulose strips followed by hybridization with the cRNA and autoradiography (*3, 9*). In each case, hybridization was only seen at that position which corresponded to the size of the yeast DNA fragment present in the hybrid.

A quantitative evaluation of the growth potentials of the various selected hybrids can be obtained by determining their burst sizes. These are compared to the burst size of a hybrid containing a DNA fragment considered to have a neutral effect on the growth of λ. Such a hybrid is λgt-EcAra6, which contains just the A, B, and D genes of the arabinose operon of *E. coli* (*10*). In the absence of arabinose, this operon is not induced and the inserted DNA segment should be neutral with respect to growth of the phage. Its burst size is about 50 (half that of a "wild-type" *red*$^+$ λ). The λ–*E. coli* hybrids selected on W3110 have a slightly higher burst size than λgt-EcAra6 (60 as compared to 50). Unexpectedly, the λ–*E. coli* hybrid selected on H560 has a higher burst size on W3110 (80) than the hybrid selected on W3110. One explanation is that some alteration occurred during the selection process on H560 which allows the affected hybrid to grow better on both H560 and W3110 than any other unaltered hybrid present in the pool. Conversely, the hybrid selected on W3110 grows very poorly on H560.

The λ-yeast hybrids selected on W3110 gave a burst size of 60 like their *E. coli* counterparts. However, the λ-yeast hybrids selected on H560 grew only moderately well on H560, with burst sizes from 10 to 20, and on W3110, with burst sizes from 30 to 60 (25 hybrids examined).

The actual nature of the effects seen for particular yeast DNA inserts on the growth of the hybrid bacteriophage remains for speculation. Although the effects seem to be innate to the fragment, no function is expressed which can be ascertained other than a cis-acting effect on growth. An important consideration related to the expression of heterologous DNA insertions is the situation of the λ–*E. coli* hybrid selected on H560, which may be the result of a rare alteration selected by the pressure of 25 growth cycles in a restrictive environment. The possibility of DNA sequence changes which provide favorable growth rates must be considered whenever hybrid bacteriophage or plasmids are grown in an unfavorable environment. An example of this is selection for the expression of a particular function by eukaryotic DNA carried into a prokaryotic host.

No λ-yeast hybrid was present in these pools of *red*$^-$ bacteriophage which had a burst size larger than two-thirds of the burst size of "wild-type" λ. When compared to the growth of λgt-EcAra6 and λgt-λB, most inserted yeast DNA segments had a neutral or slightly detrimental effect on the growth of the hybrid λ bacteriophage. These hybrids would not, therefore, be expected to compete in nature with "wild-type" λ bacteriophage.

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Use of Isolators in Recombinant DNA Research

The use of negative pressure rooms has been recommended as a means of physical containment for recombinant DNA experiments. The major drawback of such rooms is that the experimenter, because he is in contact with the potentially hazardous material, may contaminate himself and serve as a dissemination vector. By using an isolator, the experimenter can avoid any direct contact with the hazardous materials, and he needs no special safety training. Isolators in positive or negative pressure have been used for many years for the manipulation of radioactive compounds and toxic chemicals and bacteria, and they have proved efficient in preventing outside contamination.

The aim of this report is to emphasize the advantages of using isolators in recombinant DNA research. To be really safe, an isolator must be equipped with a transfer system which allows the experimenter to take material in and out without ever coming into contact with it. The Elster isolator (*1*) (Fig. 1) which we started using recently possesses a particularly elaborate transfer system (*2*, *3*): material can be taken in or out in a tightly closed container, and this container can be transferred to another isolator or directly autoclaved. The isolator is in negative pressure, with air filtrated through Hepa filters, and its internal temperatures can be regulated from 20° to 40°C so that it can be used as an incubator for plates and cultures.

Isolators of this type but in positive pressure have been used for surgical operations (*3*), the protection of immunodeficient patients (*2*, *4*), and for the

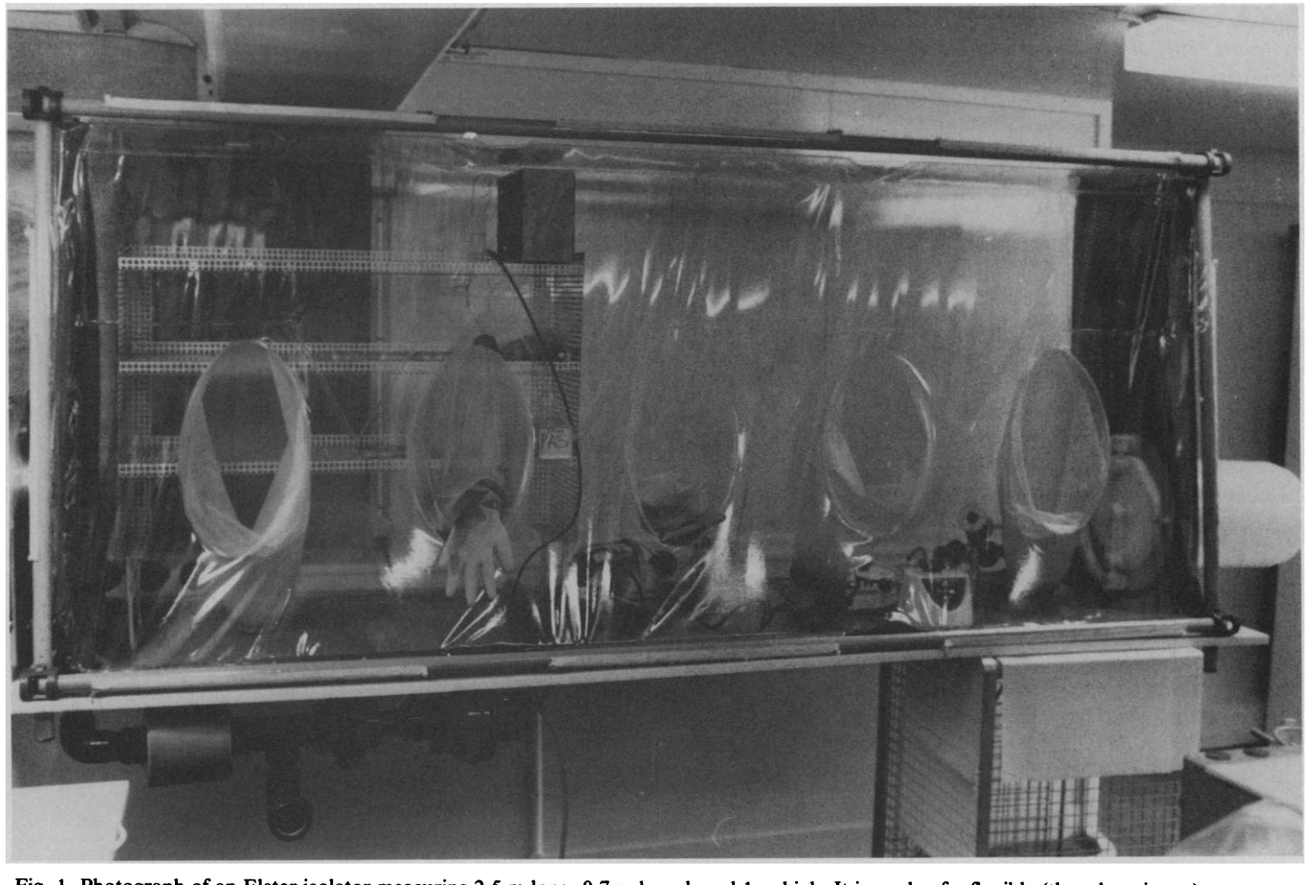

Fig. 1. Photograph of an Elster isolator measuring 2.5 m long, 0.7 m broad, and 1 m high. It is made of a flexible (though resistant) transparent plastic sheet held by a tubular frame, set up on a table with filters and machinery attached underneath. There are five regularly spaced gloves along the front, which permit manipulation in all parts of the internal volume. The isolator is maintained under negative pressure (which can be varied from 0 to −7 mm of water). The incoming and extracted air is passed through Hepa filters. Because of the limited air flow, these filters will last for many years. There are two circular openings, one on each side. The biggest one, on the left, is for taking in or out large sized equipment. The other, which is the transfer system door, shown here with a container plugged in, is used to take materials in or out during experiments. When the container with its lid is applied onto the transfer door and rotated, it triggers the isolator's door which can now be opened from inside. The lid of the container is stuck to the door in such a way that the external faces of both the lid and the door are protected from contamination. The tightly closed container can be transferred to another isolator or autoclaved (with metal containers not shown). The inlet for formol or peracetic acid is at the bottom left. The internal volume is closed and remains safe even if air extraction stops. Since plates may have to be incubated or cultures grown at temperatures ranging from 30° to 40°C, the entire isolator is thermally regulated, and the internal temperature can be varied at will. Plates are incubated on the bench, and cultures are grown on a shaking platform. A magnetic stirrer is available for cultures of 1 to 2 liters, and a small electric pump can be used to bubble air through the culture. Samples could be subjected to ultracentrifugation by sealing the opening of the ultracentrifuge in the same or another isolator while the centrifuge itself remains on the outside.