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Entrapment of a bacterial plasmid in phospholipid vesicles: Potential for gene transfer

(DNA entrapment/fusion)

ROBERT T. FRALEY, CHESTER S. FORNARI, AND SAMUEL KAPLAN

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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ABSTRACT **Entrapment of pBR322 DNA within liposomes was demonstrated by (*i*) its comigration with liposomes on Sepharose 4B columns, (*ii*) resistance of its biological activity to DNase digestion, and (*iii*) identification of plasmid DNA on agarose gels after lipid extraction. The biological activity of the liposome-entrapped plasmid was determined by transformation assays. The incubation of intact liposomes, containing entrapped pBR322, with competent *Escherichia coli* cells in the standard transformation mixture resulted in the appearance of tetracycline-resistant colonies at a frequency of 1% of the control frequency. Importantly, this frequency was unaffected by the addition of DNase to the incubation mixture, whereas transformation by free pBR322 DNA was totally eliminated after treatment with DNase.**

Recent demonstrations of the entrapment of biologically active RNA (1–3) within liposomes suggested that liposomes may also be useful for introducing specific DNA sequences into cells. This approach might offer a distinct advantage over the introduction of drugs, enzymes, and RNA into cells in that it may be possible to afford a permanent rather than temporary change in cell phenotype. In addition, the utilization of liposomes as a vector for the introduction of unique DNA sequences into recipient cells could provide a powerful method for the extension of recombinant DNA technology to both eukaryotic and prokaryotic cells that do not possess conventional means of genetic exchange. In this paper we describe the entrapment of the bacterial plasmid, pBR322, within liposomes and demonstrate the transfer of the liposome-entrapped plasmid to *Escherichia coli* strain SF8.

MATERIALS AND METHODS

Preparation of Lipids and Dye. Egg phosphatidylcholine was obtained from Sigma (purity >99%). Egg phosphatidylglycerol was synthesized from egg phosphatidylcholine by the phospholipase D-catalyzed transphosphatidylation procedure described by Dawson (4). The reaction products were separated on preparative (0.75 mm) silica gel G plates with a one-dimensional solvent system [chloroform/methanol/water, 65:35:5 (vol/vol)]. The purity of all phospholipids used in this study was confirmed chromatographically on boric acid-impregnated silica gel G plates, by the two-dimensional system of Poorthius *et al.* (5). The concentration of lipid phosphorus was determined according to the method of Bartlett (6). Arsenazo III dye was obtained from Sigma and purified as described by Weissman *et al.* (7) before use.

Preparation of Liposomes. Liposomes were prepared by a modification of the method of Deamer and Bangham (8). Thirty micromoles of egg phosphatidylcholine and 3 μmol of egg phosphatidylglycerol were dissolved in chloroform. The chloroform was removed by evaporation under N_2 gas and the lipids were resuspended in 0.5 ml of methanol, followed by addition of 10.0 ml of diethyl ether. The lipid solution was placed in a 10-ml Gastight Hamilton syringe and injected through a 23-gauge needle at a rate of 0.17 ml/min into 4.5 ml of an aqueous solution. The aqueous solution, containing either 4.3 mM arsenazo III or 350–380 μg (2×10^3 cpm of ^{32}P per μg of DNA) of pBR322 DNA per ml in buffer (5 mM Hepes/50 mM NaCl/50 mM KCl, at pH 7.35), was maintained at 60°C. The liposome suspension was allowed to cool at room temperature before application to a 1.5 × 47.0 cm Sepharose 4B column equilibrated in the above buffer. Liposomes containing DNA were treated with 20–50 μg of DNase I per ml (Worthington) for 1.0 hr at room temperature to maximize separation between liposome-entrapped and free DNA before application to the column. One-milliliter fractions were collected and assayed for the presence of liposomes (OD at 620 nm), arsenazo III (*A* at 650 nm), and plasmid [^{32}P]DNA. Radioactivity was determined by monitoring an appropriate aliquot in a Triton/toluene-based scintillant (9).

Liposome-entrapped plasmid [^{32}P]DNA was extracted by the method of Bligh and Dyer (10). A solution of 1% NaCl was used to replace the water additions during phase partitioning and washing. The combined aqueous phases were reduced in volume by evaporation under a stream of N_2 gas at 45°C and exhaustively dialyzed against 10 mM NaCl prior to use in transformation assays.

Bacterial Strains and Media. *E. coli* strain SF8, a derivative of C600, was used as recipient in all transformations. The genotype of SF8 is *thr, leu, thi, str, hsr,* and *hsm* (11). Plasmid pBR322 DNA was isolated from strain SF8.

M9 medium (12) with 0.2% Casamino Acids (Difco) was used to culture SF8 (pBR322) for plasmid isolations. A low phosphate modification of M9 with 20 mM maleic acid replacing the phosphate (13) was used for labeling pBR322 with [^{32}P]orthophosphate. LB medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of distilled water) was used to grow SF8 for all transformations. Selective plates were prepared by supplementing LB with 1.2% agar and 8 μg of tetracycline per ml.

Isolation of Plasmid DNA. The procedure for the isolation of plasmid DNA is a modification of methods described by Guerry *et al.* (14) and Humphreys *et al.* (15). Eight liters of cells were grown in M9 medium to late logarithmic phase, at which time chloramphenicol was added to 170 μg/ml and the culture was allowed to incubate overnight at 37°C. After harvesting, the cell pellet was resuspended in 0.25 M sucrose in 50 mM Tris·HCl (pH 8.0) and treated with lysozyme/EDTA, sodium dodecyl sulfate, and 5 M NaCl as described (14). Treatment of the cleared lysate with polyethylene glycol 6000 (15) yielded precipitates that were centrifuged to equilibrium in ethidium

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Abbreviation: LUV, unilamellar vesicles.

bromide/CsCl gradients (14). Generally, the yield was 12 mg of plasmid DNA.

^{32}P-Labeled plasmid DNA was isolated by the above procedure except that [^{32}P]orthophosphate was added to 22 μCi/ml one culture doubling prior to the addition of chloramphenicol (1 Ci = 3.7×10^{10} becquerels).

Transformation Procedure. The $CaCl_2$–shock method, as described by Cohen *et al.* (16), was used to prepare cells for transformation. Volumes of 0.1–0.3 ml from transformation incubation mixtures were spread onto selective agar plates consisting of LB medium with 8 μg of tetracycline per ml.

Agarose Gel Electrophoresis. Electrophoresis was performed in a 0.8% agarose (Sigma type II) slab gel in a buffer containing 160 mM Tris acetate, 80 mM Na acetate, and 4 mM Na_2EDTA, at pH 7.8. The horizontal slab gel apparatus was that of McDonnell *et al.* (17). After adding 1/5 vol of a mixture containing 33% (vol/vol) glycerol, 7% (wt/vol) sodium dodecyl sulfate, and 0.25 mg of bromphenol blue per ml, the samples were loaded onto the gel in 40-μl volumes. Electrophoresis was for 9 hr at 0.6 V/cm. The gel was stained in ethidium bromide at 3 μg/ml for 1 hr, rinsed with distilled water, and photographed with a Polaroid camera with type 665 film and an orange filter. Exposure was for 3 min with long-wave UV illumination.

RESULTS

Entrapment of Plasmid DNA in Liposomes. The ether injection method described by Deamer and Bangham (8) for producing liposomes was used for plasmid entrapment because it offers several advantages over other commonly used methods for liposome preparation. The liposomes produced by ether injection are large, unilamellar vesicles (LUV), which trap substantially more aqueous volume per μmol of lipid than either multilamellar or sonicated liposomes (8). All phospholipids (or mixtures) that produce stable liposomes can be used in this procedure (8). In addition, the absence of a sonication step facilitates the entrapment of macromolecules without loss of their biological activity.

All liposomes used in this study were prepared from a mixture of egg phosphatidylcholine and egg phosphatidylglycerol (10:1, wt/wt). This combination of lipids was chosen because fluid, negatively charged liposomes have been shown to fuse optimally with cells (18). In addition, the inclusion of negatively charged egg phosphatidylglycerol should reduce electrostatic interaction between the liposomes and DNA during vesicle formation and fractionation over molecular sieve columns. The separation of liposome-entrapped (peak I) and DNase-digested (peak II) pBR322 DNA by chromatography on Sepharose 4B is shown in Fig. 1*A*. The identity of the individual peaks was determined by comparison with the elution profiles obtained for arsenazo III dye trapped in identical liposome preparations (not shown), in which case tests for liposome integrity and arsenazo III latency described by Weissman *et al.* (7) were used. An electron micrograph of the material in peak I (Fig. 2) shows the liposomes to be heterogeneous in diameter (0.1–1.5 μm), with the average vesicle diameter being 0.23 μm. These values are in good agreement with those reported by others (8, 19) using the ether injection technique.

Typically, 2–6% (40–100 μg) of the plasmid DNA was found in association with the liposome fraction (Table 1). The similarity of the values calculated for captured volume per μmol of lipid, based on arsenazo III (6.2 μl/μmol of lipid) and pBR322 DNA (5.03 μl/μmol of lipid) entrapment, indicates that the large size of the plasmid (2.6×10^6 daltons) does not interfere with encapsulation. The values obtained compare favorably with the theoretical yield for entrapment of 7.6 μl/μmol of lipid calculated, based on a vesicle diameter of 0.23 μm.*

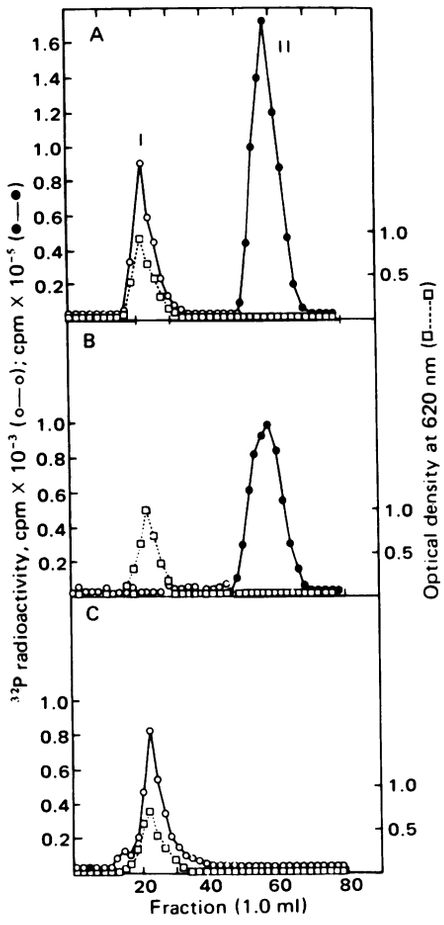

FIG. 1. Resolution of liposomes and pBR322 [^{32}P]DNA mixtures by chromatography on Sepharose 4B. Liposome/DNA suspensions (4.5 ml) were applied to the column and eluted with 0.1 M NaCl/KCl containing Hepes (5 mM), pH 7.4. Fractions (1.0 ml) were collected and monitored for the presence of lipid vesicles (□---□) and ^{32}P radioactivity (o—o, ●—●). (*A*) Typical elution profile obtained for DNase-treated liposomes containing pBR322 [^{32}P]DNA. (*B*) Elution profile obtained for DNase-treated mixture of preformed liposomes and exogenously added pBR322 [^{32}P]DNA. (*C*) Elution profile obtained for liposome-associated pBR322 [^{32}P]DNA (*A*, peak I) after retreatment with DNase.

To demonstrate that the plasmid DNA was entrapped within the liposomes and was not simply adhering to the vesicle surface, we again treated the liposome–DNA peak in Fig. 1*A* with DNase and rechromatographed the mixture on Sepharose 4B. The elution profile in Fig. 1*C* shows that the liposome-associated pBR322 DNA is resistant to DNase treatment. The possibility that plasmid DNA associated with the liposome surface may be protected from DNase digestion was tested by mixing plasmid [^{32}P]DNA and preformed liposomes. Subsequent treatment with DNase and chromatography on Sepharose 4B (Fig. 1*B*) revealed that none of the ^{32}P radioactivity was associated with the liposome fraction. Together, these results indicate that the DNA associated with peak I represents liposome-encapsulated plasmid DNA and eliminates the possibility that the association can be accounted for by adherence of DNA to the vesicle surface.

* The value for aqueous volume entrapped per μmol of lipid was calculated by using the variables 50 Å for bilayer thickness and 72 Å^2 for area per phospholipid molecule and assuming the number of phospholipid molecules in each half of the bilayer to be equal.

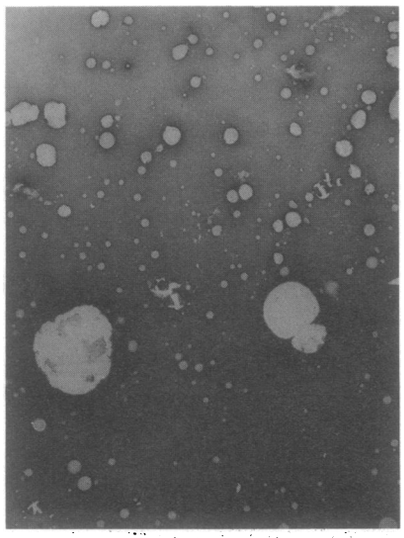

FIG. 2. Electron micrograph of liposomes, LUV (phosphatidylcholine/phosphatidylglycerol, 10:1). Samples were stained with 1% uranyl acetate and examined in a JEOL 100 C electron microscope. The average diameter of the lipid vesicles was determined to be 0.23 μm, by a Numonics (Landsdale, PA) Graphics Calculator interfaced to a Wang 600 Programmable Calculator.

Transfer of Liposomal Contents to Cell Cytoplasm. Plasmid pBR322 carries the genetic determinants for tetracycline resistance (Tet^R) and ampicillin resistance (Ap^R) (20). Therefore, the selection for either one or both of these markers provides a rapid and sensitive test for the biological activity of the liposome-entrapped plasmid DNA. We chose to assay gene transfer by selecting for Tet^R among SF8 cells exposed to liposome-entrapped plasmid DNA. All incubations of liposomes with cells were performed as described under *Materials and Methods*, and the results are reported in Table 2.

Line 4 of Table 2 presents data that indicate that gene transfer is unaffected by the presence of DNase in the incubation mixture. Line 5 shows that the same DNA, when extracted from the liposomes, becomes completely sensitive to DNase included in the incubation mixture. In addition, the data from line 6 show that the plasmid DNA, before being exposed to vesicle phospholipid, is also completely sensitive to the DNase.

The standard DNA of line 1 represents a standard, untreated plasmid preparation from which an aliquot was removed for every transformation experiment. This sample serves as an internal control for all transformation experiments because the plasmid DNA for liposome entrapment often came from different preparations.

Lines 2 and 3 of Table 2 list data that show that for a given plasmid DNA preparation, the exposure to organic solvents used to remove phospholipid from liposome-entrapped DNA does not significantly alter the biological activity of that DNA. Line 7 (Table 2) demonstrates that the incubation of empty liposomes with free plasmid DNA in the standard transformation reaction mixture does not protect the plasmid from DNase digestion. This result indicates that any plasmid DNA released from the liposomes during the incubation period will be sensitive to DNase digestion, and therefore we conclude that only liposome-entrapped pBR322 DNA is active in transformation.

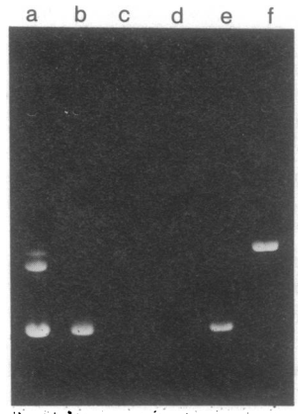

FIG. 3. Agarose-gel electrophoresis profiles of DNA isolated from phospholipid vesicles. Lanes a, b, and e, pBR322 DNA; the concentration of plasmid DNA in lane a is 5 times greater than in lanes b and e. Lane f, DNA of the plasmid RSF 1010 (5.5×10^6 daltons). Lanes c and d, DNA extracted from phospholipid vesicles.

Fig. 3 shows that the plasmid DNA is not only present within the liposomes, but also that it has sustained slight physical alterations—i.e., shifting to the open circular form. A possible cause for the conversion to the open circular form may be the heating at 60°C for 1 hr during the entrapment procedure. Fig. 4 presents an agarose gel of plasmid DNA recovered from SF8 after successful transformation with liposome-entrapped pBR322 DNA. The recovered DNA is typically in the supercoiled, closed, covalent, circular form. No pBR322 plasmid DNA could be recovered from Tet^S SF8 cells (lanes c and d, Fig. 4).

Inhibition of Transformation by Free Plasmid DNA in Presence of Liposomes. As was indicated earlier, the incubation of empty liposomes with free pBR322 DNA in a standard transformation reaction mixture plus DNase does not result in the appearance of Tet^R colonies (Table 2, line 7). For an identical incubation mixture, but one without DNase, we observed a transformation frequency less than 5% of a control experiment without liposomes. These results indicate that liposomes neither protect plasmid DNA from DNase digestion nor facilitate the uptake of plasmid DNA. In support of this last result, Fig. 5 shows that transformation of SF8 by free plasmid DNA is inhibited by increasing amounts of liposomes. At a concentration of phospholipid normally used for liposome-mediated transformation (200 μM), the frequency of transformation by free

Table 1. Encapsulation of arsenazo III and pBR322 DNA in lipid vesicles

Lipid preparation	Lipid,* μmol/ml	Captured volume,† μl/μmol lipid	% encapsulation
LUV(PC10:PG1)(A III)	8.20	6.20	2.93‡
LUV(PC10:PG1)(pBR322)	8.27	5.03	3.00

The liposome fractions, obtained after chromatography on Sepharose 4B, were pooled and analyzed for lipid phosphorus and ^{32}P radioactivity or arsenazo III absorbance (650 nm). PC10:PG1 = phosphatidylcholine/phosphatidylglycerol, 10:1.

* Micromoles of lipid per ml of aqueous phase after vesicle formation.

† Results are average of four experiments.

‡ Based on percent recovery from the Sepharose 4B column.

Table 2. Transformation by pBR322 DNA of *E. coli* SF8 cells to tetracycline resistance

DNA	DNA in reaction mixture, μg	DNase	Transformants/ μg DNA	Transformation frequency
Standard pBR322	1.40	−	6.0×10^4	4.3×10^{-4}
		+	0	0
Nonextracted, free pBR322 DNA	1.70	−	1.8×10^3	1.5×10^{-5}
		+	0	0
Extracted, free pBR322 DNA	1.50	−	6.0×10^3	5.0×10^{-5}
		+	0	0
Vesicular pBR322 (liposome-entrapped)	0.84	−	1.1×10^2	4.5×10^{-7}
		+	1.2×10^2	5.0×10^{-7}
Vesicular pBR322 extracted	0.84	−	1.0×10^4	4.0×10^{-5}
		+	0	0
pBR322 before entrapment	0.40	−	2.5×10^4	5.0×10^{-5}
		+	0	0
Empty liposomes* plus free pBR322	0.8	−	5.0×10^3	1.7×10^{-5} †
		+	0	0
Vesicles, no competent cells	0.84	−	0	0
		+	0	0

Standard pBR322 DNA was a stock preparation that was included in every transformation experiment to serve as an internal control. Vesicles without competent cells were always included to control for contamination from the vesicle preparation. Transformation frequencies are expressed as the number of transformants per total number of viable cells. The results shown are the average of at least three experiments.

* Equivalent to 210 nmol phospholipid per ml of transformation incubation mixture.

† Control transformation frequency for these experiments was 5.7×10^{-4}.

DNA is reduced about 95%. Throughout these experiments, cell viability was tested and found not to be affected by the addition of liposomes (unpublished data).

DISCUSSION

This report provides a demonstration of the entrapment of a specific DNA molecule, pBR322, within liposomes. We have also demonstrated that liposomes can be used to deliver entrapped materials to the cytoplasm of bacterial cells. Entrapment of the plasmid DNA was demonstrated by its comigration with liposomes on a Sepharose 4B column, resistance to DNase treatment, and identification on agarose gels. The biological activity of the liposome-entrapped plasmid was determined by transformation assays after removal of the lipid by extraction.

The incubation of intact liposomes, containing entrapped pBR322, with competent cells in the standard transformation reaction mixture (66 mM Ca^{2+}) results in the appearance of Tet^R colonies at a low frequency (≈1% that obtained for free pBR322 DNA). However, this frequency is unaffected by the addition of DNase to the reaction mixture, whereas transformation by free pBR322 DNA is totally eliminated by treatment with DNase. The experiments carried out with empty liposomes

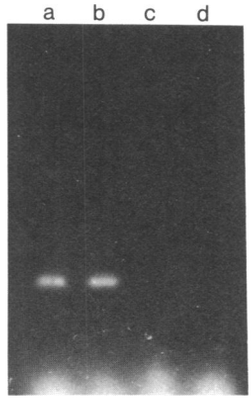

FIG. 4. Agarose-gel electrophoresis profiles of plasmid DNA isolated from individual colonies. *E. coli* strain SF8, SF8 (pBR322), and SF8 acquiring Tet^R through exposure to vesicular pBR322 DNA were examined for the presence or absence of plasmid DNA by a modified plasmid isolation procedure. Lane a, pBR322 DNA isolated from a colony transformed with free pBR322 DNA; lane b, pBR322 DNA isolated from a colony transformed with vesicular pBR322 DNA; lanes c and d, colonies from strain SF8 carrying no plasmid and, therefore, Tet^S.

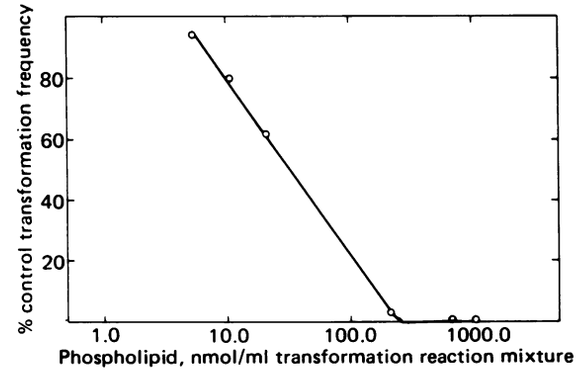

FIG. 5. Effect of empty liposomes on the transformation of SF8 by free pBR322 plasmid DNA. Dilutions of this liposome preparation were added to a series of test tubes containing the standard transformation reaction mixture. Transformation frequencies were expressed as a percentage of control sample containing no liposomes.

and free pBR322 DNA indicate that liposomes do not promote the cellular uptake of the plasmid, nor do they protect the plasmid from DNase digestion (Fig. 5 and Table 2).

It is unlikely that the transformation of SF8 exposed to liposome-entrapped pBR322 occurs as the result of DNA released from the liposomes after plating of the reaction mixtures for the following reasons: (*i*) the liposomes are quite stable and leakage of DNA has not been detected (Fig. 1*C*), (*ii*) there is a marked dilution of recipient cells and liposome-entrapped DNA, making contact unlikely, (*iii*) DNase is present on the surface of the plates, and (*iv*) phenotypic expression is not permitted because tetracycline is present at the time of plating. Furthermore, in other experiments (unpublished data) plasmid DNA was added to plates of the outgrowth medium containing competent cells,, and, after incubation for 1.5 hr at 37°C, no Tet^R colonies were observed.

Jones and Osborn (21, 22) have shown that the incubation of phospholipid vesicles with *Salmonella typhimurium* cells results in the uptake of ≈10% of all vesicle components, including cholesterol oleate and lipopolysaccharide. They suggested that the uptake of intact vesicles was occurring, rather than a phospholipid exchange mechanism. Vesicle uptake was shown to be dependent on the lipopolysaccharide composition of the recipient cells and the presence of 10 mM Ca^{2+}. The mechanism of the uptake process was interpreted as direct fusion of the lipid vesicles with the outer membrane of the cells. A similar demonstration has been reported by McIntyre and Bell (23), who demonstrated the uptake of 1-oleoglycerol-3-phosphate by a deep rough mutant of *E. coli*. Although the mechanism of lipid uptake in this study is unclear, the observation that oleoglycerol phosphate binding and uptake is enhanced 5- to 6-fold in the deep rough derivative as compared to the wild-type strain argues for the direct interaction of oleoglycerol phosphate with the outer membrane. In this regard, preliminary experiments (unpublished data) with a deep rough mutant of *E. coli* have shown that the frequency of liposome-entrapped plasmid DNA transformation is at least one order of magnitude higher than is found with the wild-type strain.

It is tempting to speculate, in view of the above consideration, that the appearance of Tet^R colonies after incubation of intact liposomes with competent cells results from the fusion of liposomes with cells and release of the plasmid DNA into the cell cytoplasm or periplasmic space. The low frequency of transformation by liposome-entrapped pBR322 in comparison with free pBR322 might be explained by the fact that transformation by liposome-entrapped pBR322 would require both the interaction of the liposome with the cell and subsequent entry of the plasmid. It is apparent from a number of studies (21, 22, 24, 25) that only a small fraction (1–5%) of the liposomal population actually interacts with cells. Assuming that a maximum value of 10% of the liposomes productively interact with SF8 cells and that only 20% of the liposome population contains pBR322 DNA,† the transformation efficiency calculated for liposome-entrapped pBR322 would be quite similar to that observed for free pBR322 DNA.

The procedure described in this study has broad applicability for extending current recombinant DNA technology to both prokaryotic and eukaryotic cells that lack conventional genetic exchange systems. With respect to prokaryotes, the usefulness of this technique depends on transforming otherwise nontransformable strains by use of liposome-entrapped DNA or increasing the frequency of transformation in strains that are already transformable at low frequency. However, the demonstration of liposome-mediated transformation in bacterial species that are otherwise nontransformable is further complicated by the restriction of the plasmid DNA by the host cell as well as efficiencies of genetic expression of the DNA within the host cell. In addition, the use of recombinant plasmid vectors requires, at this time, approval for use in new hosts. For these reasons, we have initially developed this method within a well-known system which circumvents all the above-mentioned problems.

The ultimate utility of this approach will depend on increasing the efficiency of liposome-promoted gene transfer. As mentioned above, the use of deep rough mutants of *E. coli* with the liposome-entrapped DNA appears to yield transformation frequencies greater than those observed for the standard system.

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† The percentage of liposomes containing pBR322 was determined from the experimental value of 2.9 μg of DNA trapped per μmol of lipid (average of four experiments). The average number of plasmid DNA molecules per liposome was ≈0.25, and the percentage of liposomes containing pBR322 was calculated by using the Poisson distribution.