JOURNAL OF BACTERIOLOGY, Sept. 1982, p. 1078–1085
0021-9193/82/091078-08$02.00/0
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Vol. 151, No. 3

Small DNA-Free Liposomes Stimulate Transfection of *Streptomyces* Protoplasts

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Received 10 March 1982/Accepted 27 May 1982

DNA of the bacteriophage φC31 was rendered DNase resistant by entrapment in liposomes. Liposome-entrapped φC31 DNA transfected *Streptomyces* protoplasts in the presence of 50% polyethylene glycol (PEG), providing a potential alternative route to conventional PEG-mediated transfection of protoplasts. However, probably partially because of low entrapment of DNA, this system did not result in an effective increase in transfection efficiency over the conventional transfection procedure. A more effective use of liposomes for stimulating transfection was provided by the discovery that supernatants obtained during the washing of DNA-free liposome preparations stimulated PEG-mediated transfection of protoplasts. This effect appeared to involve small (0.1- to 0.3-μm diameter) poorly sedimented liposomes. It was most effective (more than 100-fold stimulation) with positively charged liposome supernatants and high (about 50% [wt/vol]) PEG concentrations. Stimulation of transfection was also observed with cloning ligation mixtures containing φC31 DNA as the vector. Transformation by plasmids (but not by chromosomal DNA fragments) was also significantly more efficient in these conditions than in conventional protoplast transformation.

When phospholipids are dispersed in water, vesicles termed liposomes are formed, which contain water, together with any dissolved solutes, inside their bilayered (or multilayered) lipid membrane. A great variety of biologically active compounds and structures can be included within the liposomes, ranging from small molecules to macromolecules (for reviews, see references 15 and 29) and even chromosomes (28), chloroplasts (14), and virus particles (37). Fusion of liposomes with cells, therefore, provides a route for the introduction of such materials into cells. The use of liposomes for the introduction of DNA into cells could be relevant to recombinant DNA technology, and studies of liposome encapsulation of DNA and its subsequent delivery into animal cells (11, 12, 38), plant protoplasts (23, 24, 27, 31) and bacteria (13, 25) have recently been published.

In *Streptomyces* sp. the availability of techniques for the transformation or transfection of plasmid or bacteriophage DNA into protoplasts (4, 20, 21, 32) has permitted the development of plasmid- and phage-based gene cloning systems (3, 33, 36) in this important group of differentiating and antibiotic-producing bacteria. However, the efficiency of the existing transfection process (10^{-6} to 10^{-5} per DNA molecule; 32, 34) is lower than frequencies of plasmid transformation (up to 10^{-4}; 4), hindering shotgun cloning experiments with phage vectors. A route to increased transfection efficiency was suggested by the experiments of Makins and Holt (25), who showed that liposome-entrapped chromosomal DNA transformed a very high proportion of *Streptomyces* protoplasts in the presence of >40% polyethylene glycol (PEG). We, therefore, tried to apply this use of liposomes to transfection by DNA of the temperate phage φC31, which has been developed in our laboratory as a cloning vector (7, 8, 33, 34). Arising from this investigation has been the discovery of a new, efficient, and easily applied "liposome-assisted" transfection system which also improves transformation by plasmid DNA.

MATERIALS AND METHODS

Bacteria. *Streptomyces coelicolor* A3(2) strains used were M145 ($SCP1^-$ $SCP2^-$) and 2709 (*hisA1 proA1 argA1 cysD18 uraA1 strA1*, $SCP1^-$ SCP2), and *S. lividans* 66 strains used were 1326 (wild type) and TC52 (containing pIJ41).

DNA preparations. DNA of φC31 *c*1, a clear-plaque mutant of the temperate bacteriophage φC31 (22), was extracted as described elsewhere (9), and pIJ502, the *Escherichia coli* plasmid form of φC31 cts1::pBR322ΔW17 (5) was extracted by cesium chloride-ethidium bromide centrifugation of a cleared lysate (as described elsewhere [10] but with replacement of the Brij-deoxycholate mixture in the detergent mixture by 0.1% Triton X-100 for the lysis of spheroplasts). Plasmid pIJ41 (C. J. Thompson, T. Kieser, J. M. Ward, and D. A. Hopwood, manuscript in

preparation) was prepared by the large-scale method of reference 2, and chromosomal DNA was prepared as described in reference 8.

Media and reagents. P and R2 media were prepared as described elsewhere (18); R2YE (8) was R2 medium containing 0.5% yeast extract, and minimal medium (MM) has been described previously (16). TE buffer contained 0.01 M Tris-hydrochloride and 0.001 M EDTA (pH 8.0). G buffer (25) contained 0.015 M NaCl, 0.0015 M trisodium citrate, 0.28 M sucrose, 0.001 M $CaCl_2$, 0.1 M threonine, and 0.1 M histidine (pH 7.0). LSM (liposome sedimentation medium) contained 0.7 M KCl and 10% ethanol. PEG (molecular weight, 1,000) was from BDH Chemicals, Poole, Dorset, England. DNase I was from Sigma Chemical Co., St. Louis, Mo. L-α-phosphatidylcholine was usually from Sigma (type IX-E [no. P-8640]; the manufacturer's estimate of phosphatidylcholine content based on choline determination, approximately 60%) or (for the experiment with purified lecithin) from Lipid Products, South Nutfield, near Redhill, Surrey RH1 5PG, England (egg lecithin grade I, no. 667). Dicetyl-phosphate (high purity based on manufacturer's melting point determination) and stearylamine (>98% pure) were from P. L. Biochemicals, Inc., Milwaukee, Wis.

Preparation of liposomes. Neutral liposomes were prepared from L-α-phosphatidylcholine, negatively charged liposomes were prepared from L-α-phosphatidylcholine and dicetylphosphate (ratio, 5:1 [wt/wt]), and positively charged liposomes were prepared from L-α-phosphatidylcholine and stearylamine (ratio, 20:1 [wt/wt]). Liposomes containing DNA were made by the hand-shaken method of Bangham et al. (1): approximately 3 mg of total lipid (unless stated otherwise) was dissolved in chloroform and dried in a thin film by rotary evaporation, under vacuum (using a water pump) at 55°C. Subsequent manipulations were at room temperature unless stated otherwise. DNA dissolved in 0.3 ml of G buffer was shaken manually for about 1 min with the lipid film and spun on the rotary evaporator for 10 min. The phospholipids formed vesicles in the aqueous medium, entrapping some of the DNA. Liposomes were diluted with 4 ml of LSM, collected by centrifugation at about 6,000 rpm for 15 min, and washed again in LSM. Finally, the pellet was treated with 10 U of DNase for 1 h at 37°C. Empty liposomes were prepared by the same method, except that DNA was not added to the G buffer. In those cases where the supernatant fluid remaining after liposome sedimentation was used, it was obtained after diluting the empty liposome suspension with 1 ml, instead of with 4 ml, of LSM. The term liposome supernatant fluid (LSF) refers to such supernatant fluids.

Liposome-mediated transfection (transfection of protoplasts by DNA included in liposomes). Protoplasts from *S. lividans* were prepared as described in reference 18 (but lytic enzyme number 2 was omitted). Transfection was performed by resuspending centrifuged protoplasts in the drop of liquid remaining after pouring off the supernatant fluid and adding the pellet from a preparation of liposomes containing DNA, followed immediately by 0.8 ml of 50% PEG 1000 in P medium. After 5 min, the mixture was diluted with 4 ml of P medium, centrifuged, and suspended in a small volume of P medium. Serial dilutions of the protoplasts were then plated in soft agar overlays on R2YE medium containing *S. lividans* spores as indicator. The individual transfection events were detected by the formation of plaques after overnight incubation. As a control procedure, free φC31 DNA was added to *S. lividans* protoplasts followed by 0.8 ml of 50% PEG in P medium as previously described (32).

Isolation of phage DNA from liposomes. The phage DNA included within liposomes was isolated by two chloroform extractions, using TE buffer in the aqueous phase. The residual chloroform was removed by evaporation. The biological activity of the recovered DNA was determined by standard transfection (8).

Liposome-assisted transfection and transformation (Transfection/transformation of protoplasts by free DNA in the presence of liposome supernatant fluid). DNA diluted in 0.1 ml of LSF was added to the protoplast suspension and mixed immediately with 0.5 ml of 60% PEG. Transfection events were detected as described above. For transformation experiments, the transformation mixture was diluted to give 1 ml and then spread on 10 R2YE plates to allow sporulation. As a control procedure, standard transformation by plasmid DNA was performed as previously described (8). Plasmid transformants were recognized by the appearance of pocks (4) in the confluent culture of the regenerated protoplasts. Chromosomal transformants were sought by harvesting total spores from the regenerated cultures and plating them on appropriately supplemented selective MM.

Calculation of frequencies. Transfection/transformation frequency is expressed as the number of transfectants/transformants per DNA molecule. The φC31 genome comprises 41.2 kilobases (kb; 6), giving a molecular weight of 27.5×10^6. One microgram of φC31 DNA, therefore, contains 2.2×10^{10} molecules. Similarly, 1 μg pIJ502 DNA (38.2 kb: 5) contains 2.3×10^{10} molecules and 1 μg of pIJ41 DNA (14.8 kb; C. J. Thompson and J. M. Ward, personal communication) contains 6.1×10^{10} molecules.

Protoplast viable counts. Samples of each transfection/transformation mixture were serially diluted in P medium and spread on R2YE to give protoplast viable counts.

RESULTS

Transfection by φC31 DNA in neutral liposomes. φC31 DNA trapped in neutral liposomes transfected *S. lividans* protoplasts in the presence of 50% PEG (Table 1). The number of transfection events obtained from a given amount of starting DNA was similar to that obtained with the conventional transfection system (here using 50% PEG), but transfection by DNA trapped in liposomes was resistant to DNase. DNA reisolated from liposomes was assayed biologically (by standard transfection) and physically (by agarose gel electrophoresis against various standards). From a liposome preparation (made in the presence of 250 ng of DNA) able to give 4.5×10^3 plaques, enough DNA was extracted to give 3×10^2 plaques by normal transfection, corresponding to the num-

TABLE 1. Liposome-mediated transfection of *S. lividans* 66 strain 1326 protoplasts by φC31 *c*1 DNA[a]

Relevant additions	ng of DNA	DNase	No. of plaques	Transfection frequency[b]
Free DNA	250	−	1.5×10^4	2.66×10^{-6}
(control)		+	0	$<10^{-10}$
DNA putatively	(5)[c]	−	1.2×10^4	Not calculable
trapped in lip-osomes		+	1.6×10^4	Not calculable
DNA plus	250	−	1.6×10^4	2.84×10^{-6}
empty lipo-somes[d]		+	66	1.17×10^{-8}

[a] A total of 10^9 protoplasts, 10^9 neutral liposomes, and 0.8 ml of 50% PEG 1000 (final concentration, ca. 46%) were used in each transfection.

[b] Plaques per DNA molecule.

[c] A 250-ng amount of DNA was added to the lipid film, but the fraction entrapped was not determined in this experiment: the value given was based on data from another experiment (see the text).

[d] Note that only the centrifuged liposome pellet was used (compared with later experiments with liposome-assisted transfection).

ber of plaques given by 5 ng of DNA in a control transfection. No DNA could be seen by agarose gel electrophoresis, using ethidium bromide staining (in conditions where 12.5 ng of φC31 DNA was just detectable). We concluded that about 2% of the 250 ng of DNA used in preparing the liposomes had been entrapped, as a result of which it became 15 times more efficient in transfection than was free DNA.

Transfection by the supernatant obtained after sedimenting liposomes plus φC31 DNA. Since only about 2% of the φC31 DNA was entrapped during liposome preparation, we anticipated that 98% of it should be detectable biologically in the supernatant remaining after sedimentation of the liposomes. The number of transfectant plaques (3×10^5) obtained with this supernatant (Table 2) proved to be significantly higher than the expected value of 1.7×10^4, as if transfection were being stimulated by a component of the supernatant. Most of these transfection events were eliminated by DNase, suggesting that free DNA was involved in most of this extra activity. The high frequency of transfection was manifested only after larger liposomes had been removed by centrifugation (Table 2). Moreover, it was not due to the LSM in which the liposomes were washed. The addition of LSM to the standard transfection mixture (with 20% PEG) caused little increase in efficiency, but when the LSF from a DNA-free neutral liposome preparation was added and the concentration of the added PEG solution was increased to 40%, a marked improvement (about 10-fold) of transfection efficiency was obtained. It, therefore, seemed possible that the active agent in the liposome supernatant might be small liposomes which had failed to sediment. Since the addition of DNase to the transfection mixture virtually eliminated this transfection, entrapment of φC31 DNA was not involved.

In several experiments, primary liposome preparations, instead of being centrifuged to give pellet and LSF fractions, were sonicated for various lengths of time (at room temperature). The resulting preparations strongly stimulated transfection (in the presence of 40% PEG), but the effect was less reproducible and less marked than with LSF preparations.

Effect of charge on liposome-assisted transfection. An experiment testing the effects of negative charge (resulting from the incorporation of a small proportion of dicetylphosphate into the liposomes) and positive charge (resulting from the incorporation of stearylamine) is shown in Table 3. A striking stimulation (more than 100-fold over a control transfection without liposomes) was observed with the positively charged LSF, and an intermediate level of stimulation was seen with the negatively charged LSF. (Neither pelleted neutral liposomes nor the supernatants from neutral, negatively charged, or positively charged liposomes significantly affected the viability of protoplasts, eliminating this as an explanation of the effects on transfection [data not shown].)

TABLE 2. Biological activity of the φC31 *c*1 DNA present in the supernatant fluid of neutral liposomes

Relevant addition	ng of DNA	DNase	No. of plaques[a]	Transfection frequency
Free DNA	250	−	1.7×10^4	3.1×10^{-6}
(control)		+	0	$<10^{-10}$
DNA lipo-somes (un-washed primary liposome preparation)	250	−	2.7×10^4	4.9×10^{-6}
		+	0.69×10^4	Not applica-ble
DNA liposomes	(5)[b]	−	1.44×10^4	1.3×10^{-4b}
(pellet)		+[c]	Not done	
DNA liposomes	(245)[b]	−	30×10^4	5.6×10^{-5b}
(supernatant fluid)		+	1.8×10^4	Not applica-ble
DNA liposomes (sum)	250	−	31×10^4	5.58×10^{-5}

[a] Using 0.8 ml of 50% PEG 1000 (final concentration, ca. 43%), except in the free DNA control, in which 0.5 ml of 20% PEG 1000 (final concentration, ca. 16.3%) was added.

[b] Assuming 2% entrapment; see the text.

[c] Although this was not done in the experiment shown, no significant effect of DNase was found in many other tests with liposome-entrapped DNA pellets.

Effect of PEG concentration on liposome-assisted transfection. In agreement with previous results (32), transfection in the absence of LSF showed an absolute PEG dependence (within the sensitivity of the experiments) and was maximally stimulated when the final PEG concentration was about 25% in the transfection mixture (Fig. 1). Different curves of PEG dependence were obtained when LSF preparations were present (Fig. 1). The highest PEG concentration (60%) used with positively charged LSF gave the highest frequency, and in the case of neutral LSF, a low level of transfection (4.2×10^{-8} per DNA molecule) was obtained without PEG.

Factors affecting liposome-assisted transfection with positively charged LSF. Since the highest transfection frequencies were obtained with positively charged LSF preparations, further experiments were focused on their use. A final concentration of 50% PEG (0.5 ml of 60% PEG) was used in subsequent experiments (unless stated otherwise).

(i) Effect of DNA concentration. The number of plaques increased in proportion to the amount of DNA added, up to 25 ng of DNA (equivalent to 5.5×10^8 DNA molecules). At this point, the ratio of DNA molecules to viable protoplasts was about 32 to 1, and 1 in 220 DNA molecules and 1 in 7 viable protoplasts gave plaque formation. When the DNA/protoplast ratio was higher than this, the efficiency gradually fell, until at very high ratios the absolute number of plaques was somewhat reduced.

(ii) Effect of the number of protoplasts. With a nonsaturating amount of DNA (2.5 ng), the same numbers of plaques (2.2×10^5 to 2.6×10^5) were obtained when the number of protoplasts was varied between 1×10^7 and 3×10^8.

(iii) Effect of varying the amounts of LSF added. In our standard conditions for liposome-assisted transfection, the frequency was not limited by the amount of LSF added (either for positively charged or neutral preparations). Reducing the volume of supernatant from 100 to 10 μl in conditions of DNA limitation produced no reduction in the number of plaques. In experiments with sonicated preparations of liposomes, significant stimulation was obtained with 100 μl of 100-fold dilutions.

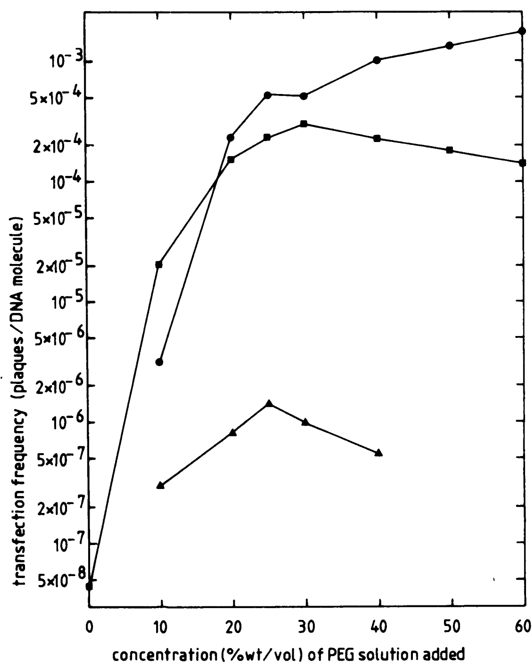

FIG. 1. Effect of PEG 1000 concentration on transfection by φC31 DNA in the presence and absence of neutral and positively charged LSF preparations. The PEG solution (0.5 ml) was added to a mixture consisting of a protoplast pellet and 25 μl of LSM (▲) or neutral (■) or positively charged (●) LSF containing φC31 *c*1 DNA. The volume of the protoplast pellet, although not accurately measurable, was greater than the volume of LSF or LSM added; we have, therefore, not attempted to estimate the final PEG concentrations. No transfection was detected in the absence of PEG either with positively charged LSF (frequency $<2 \times 10^{-7}$ per DNA molecule) or with no LSF present (frequency $<10^{-8}$ per DNA molecule).

TABLE 3. Effect of liposomal charge on transfection of *S. lividans* 66 protoplasts by free φC31 *c*1 DNA in the presence of LSF

LSF added to 2.5 ng of free DNA	Transfection frequency	Stimulation factor over control
None (control)[a]	2.7×10^{-5}	
Neutral[b]	6.6×10^{-5}	2.4
Negatively charged[b]	59×10^{-5}	22
Positively charged[b]	426×10^{-5}	158

[a] A 0.5-ml amount of 25% PEG (final concentration, ca. 21%).
[b] A 0.5-ml amount of 60% PEG (final concentration, ca. 50%) and 100 μl of LSF.

Evidence that small liposomes are involved in liposome-assisted transfection. (i) Effect of high-speed centrifugation of a liposome preparation. An unfractionated liposome preparation suspended in LSM was subjected to high-speed centrifugation (36,000 rpm, Beckman 40 rotor, 1 h, 15°C). The high-speed supernatant gave only 6.5% (14-fold over control transfection) of the stimulatory activity obtained with a low-speed supernatant from the same initial liposome preparation (214-fold over the control transfection). This result indicates that particulate material was involved in the stimulatory effect.

(ii) Effect of pure lecithin. When pure (>99%) lecithin was substituted for the 60% pure materi-

TABLE 4. Liposome-assisted transformation of *S. lividans* 66 strain 1326 protoplasts by pIJ41

Relevant addition	Transformation frequency[a]		Mean stimulation over control
	Expt A	Expt B	
No DNA	Undetectable	Undetectable	Not applicable
0.1 ng of DNA (control)[b]	1.73×10^{-5}	0.69×10^{-5}	Not applicable
0.1 ng of DNA plus neutral LSF[c]	2.60×10^{-5}	1.69×10^{-5}	2
0.1 ng of DNA plus negatively charged LSF[c]	3.0×10^{-5}	0.17×10^{-5}	<1
0.1 ng of DNA plus positively charged LSF[c]	4.34×10^{-4}	1.82×10^{-4}	26

[a] Pocks per DNA molecule in two separate experiments (A and B).
[b] Conventional transfection as in reference 8.
[c] Using 0.1 ml of LSF and 0.5 ml of 60% PEG (final concentration, ca. 50%).

al routinely used in the preparation of positively charged LSF, a 50-fold stimulation of transfection by the LSF was still obtained, tending to eliminate a major role for impurities present in the 60% pure lecithin in stimulating transfection and strengthening the view that the effect involved liposomes. However, a control preparation from impure lecithin was three times more efficient than that from pure lecithin, indicating either differences in the interaction of liposomes of slightly different composition with DNA and protoplasts or an accessory effect of a free contaminant present in the impure lecithin.

(iii) Use of supernatants of successive liposome centrifugations. It seemed reasonable to postulate that the stimulatory effect of liposome supernatants was due to very small and less easily sedimented liposomes (indeed, lipid vesicles of 0.1- to 0.3-μm diameter were detectable by electron microscopy; data not shown). Vigorous resuspension of the liposome pellet should have generated further small liposomes so that many successive supernatants should have continued to elicit high transfection frequencies. This did, indeed, occur through at least four successive resuspensions, strengthening the view that liposomes were implicated.

Liposome-assisted transfection by supercoiled covalently closed circular φC31 DNA. Suarez and Chater (32) described a φC31::pBR322 hybrid able to act as a phage in *Streptomyces* sp. and as a plasmid in *E. coli*. Preparations of the plasmid from *E. coli* could transfect *S. lividans* protoplasts with an efficiency 10 to 100 times lower than that obtained with the linear DNA isolated from phage particles (32). The reason for the lower efficiency is not clear. Transfection by such plasmid DNA preparations (the derivative that we used [pIJ502] was a deletion mutant of the original chimera; 5) was significantly (63-fold) increased by adding positively charged LSF. A lower, but definite stimulation (6.5-fold) was obtained with a negatively charged preparation, but little or none was obtained with a neutral preparation. It was not excluded that minor open circular or linear components of the plasmid preparation could have been responsible for the transfection events observed.

Liposome-assisted transformation by plasmid DNA. The result with supercoiled φC31 DNA preparations suggested that transformation by *Streptomyces* plasmid covalently closed circular DNA would also be stimulated by positively charged LSF. Table 4 shows two such experiments, in which a 26-fold stimulation of transformation by pIJ41 was observed when positively charged LSF was used (similar results were obtained with other SLP1 derivatives; data not shown). No significant stimulation by neutral or negatively charged LSF preparations was detected.

Stimulation of chromosomal transformation by liposome supernatants. Makins and Holt (25) obtained high frequencies of transformation for *S. coelicolor* chromosomal markers, using liposome-entrapped DNA. In two attempts, we obtained no detectable transformants (less than 10^{-7} per viable protoplast) with or without the use of the three kinds of LSF preparations (1 μg of M145 donor DNA was used with 2709 as the recipient, and the markers tested were *proA*$^+$, *hisA*$^+$, *argA*$^+$, *cysD*$^+$, and *uraA*$^+$; with positively charged liposomes, 0.1 and 5 μg of donor DNA were also used).

Liposome-assisted transfection by cloning ligation mixtures. An important potential use of LSFs, is in stimulating the uptake of chromosomal DNA fragments ligated to restriction enzyme-cleaved φC31 vector DNA. In each of three separate experiments (details of which will be published elsewhere), using ligation mixtures containing 0.8 to 1.0 μg of vector DNA and 0.2 μg of foreign DNA, liposome-assisted transfection gave more plaques than the control procedure (25% PEG and no liposomes). The increments were ×10, ×11, and ×51. The fraction of plaques in which inserted foreign DNA was present was measured in the latter two experiments at 3 and 38%: in neither case were these values affected by the mode of transfection used.

Effect of competing DNA on liposome-assisted transfection. Transfection with cloning ligation mixtures using φC31 vectors is often 3 to 4 orders of magnitude less efficient (per vector DNA molecule) than with the starting vector DNA (our unpublished data). Thus, a shotgun experiment in which 10^4 plaques were required would necessitate the use of microgram amounts of vector DNA. It was, therefore, important for cloning purposes to determine the effect of excess biologically inactive φC31 DNA on transfection efficiency. The number of plaques obtained with 20 ng of φC31 DNA was reduced about twofold when 800 ng of largely inactivated φC31 DNA (which had been cut by a restriction enzyme, mixed with 200 ng of restriction enzyme-treated *Streptomyces* DNA, and ligated) was present in the transfection mixture and about 10-fold when 1 μg of *S. coelicolor* DNA was added.

Storage of liposomes. Positively charged LSF preparations kept at 4°C or −70°C showed no loss of stimulatory activity after 18 days of storage. The starting activity was reduced by 50% after storage at room temperature for 18 days.

DISCUSSION

Initially, we set out to improve previous transfection efficiencies by encapsulating φC31 DNA in liposomes and fusing these to *Streptomyces* protoplasts, as was done for chromosomal DNA by Makins and Holt (25). Unlike the latter authors, we were unable to make good liposome preparations from *Streptomyces* membranes and, therefore, resorted to heterologous lipids available commercially. The hand-shaken procedure gave only a few percent entrapment of φC31 DNA, but this DNA was able to transfect with a frequency about 15-fold higher (per DNA molecule entrapped) than was obtained with naked phage DNA in control experiments. Thus, liposome entrapment might prove a useful way of increasing transfection frequencies if higher entrapment rates could be achieved. This should be possible with the reverse-phase system of liposome preparation, in which more than 50% of added DNA can be entrapped (35).

The discovery that the supernatants obtained during liposome preparation stimulated transfection (usually more than 100-fold with positively charged preparations), when added to DNA-protoplast mixtures in the presence of 50% PEG, offered an experimentally more convenient solution to the problem of low transfection frequencies, and this novel effect was, therefore, examined in more detail. The term liposome-assisted transfection has been used to describe the effect. We justify the conclusion that liposomes are involved as follows. First, although the PEG dependence may differ in detail from that seen for protoplast fusion, a requirement for high (50% [wt/vol]) PEG concentrations is similar to that for protoplast fusion (17, 19) and for transformation by liposome-entrapped chromosomal DNA (25) and much higher than the 20 to 25% giving optimum transfection (20, 21, 32) or transformation (4) of protoplasts in the absence of liposomes. Second, the stimulatory component was particulate, in that high-speed-centrifuged supernatants lost most of their activity. Third, the strong stimulation was still obtained when highly purified lipids were used in the preparation, ruling out the involvement of a major soluble impurity. Fourth, the liposome pellet obtained during liposome preparation (consisting mainly of larger liposomes and often itself inhibitory to the supernatant effect) repeatedly generated stimulatory activity in conditions where small liposomes would again be formed (i.e., by vigorous resuspension in an aqueous medium). Fifth, small liposomes could be seen in the supernatants by electron microscopy (data not shown).

The mechanism by which the stimulation occurs is not clear, even at a gross level, but we may speculate that PEG causes the small liposomes to fuse with relatively large protoplasts, bringing about surface changes that favor DNA uptake, and that the introduction of positive charge onto the liposomes causes DNA molecules to bind to them and, thus, to be brought into contact with the areas of the protoplast surface at which fusion with liposomes is occurring. It is difficult to account for the occasional inhibitory effect of larger liposomes; perhaps they may "mop up" smaller liposomes through liposome-liposome fusions. The stimulation caused by neutral and negatively charged liposomes may also result from their fusion with the protoplasts, the effect on transfection being lower than with positively charged preparations because DNA is not concentrated in the areas of fusion. It would be of some interest to find out whether neutral liposomes, which caused a low level of PEG-independent transfection, also bring about PEG-independent protoplast fusion. The effect of negatively charged liposomes was not tested in the absence of PEG, but they have been observed to bring about fusion between mammalian cells (30).

Since liposome-assisted transfection is effective with cloning ligation mixtures containing φC31 vector DNA (albeit less effective than with uncleaved φC31 DNA), it may be used to facilitate gene cloning in *Streptomyces* sp. It should now be possible, using only a few micrograms of φC31 vector DNA, to obtain clones for essentially the whole *Streptomyces* genome. (Suitable vectors are described in reference 8.) However, it will be necessary to take account of the

somewhat reduced transfection efficiencies obtained in transfections involving microgram quantities of DNA. The LSF effect was also manifested on plasmid transformation (Table 4), so that transformation frequencies of 10^7 per μg could be obtained more reproducibly than with the previous procedure (C. J. Thompson and J. M. Ward, personal communication).

ACKNOWLEDGMENTS

M.R.R. (on leave from the University of Oviedo, Oviedo, Spain) was supported by a postdoctoral fellowship from the Spanish Ministry of Education.

We are grateful to Bob Horne for electron microscopy of LSF preparations, Charles Thompson and Judy Ward for help with plasmid transformations, Celia Bruton for help with experiments involving ligation mixtures, and David Hopwood and John Makins for numerous technical discussions and for comments on the manuscript.

LITERATURE CITED

1. **Bangham, A. D., M. M. Standish, and J. C. Watkins.** 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. **13**:238–252.
2. **Bibb, M. J., R. F. Freeman, and D. A. Hopwood.** 1977. Physical and genetical characterisation of a second sex factor, SCP2, for *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. **154**:155–166.
3. **Bibb, M. J., J. L. Schottel, and S. N Cohen.** 1980. A DNA cloning system for interspecies gene transfer in antibiotic-producing *Streptomyces*. Nature (London) **284**:526–531.
4. **Bibb, M. J., J. M. Ward, and D. A. Hopwood.** 1978. Transformation of plasmid DNA into *Streptomyces* at high frequency. Nature (London) **274**:398–400.
5. **Chater, K. F., C. J. Bruton, W. Springer, and J. E. Suarez.** 1981. Dispensable sequences and packaging constraints of DNA from the *Streptomyces* temperate phage φC31. Gene **15**:249–256.
6. **Chater, K. F., C. J. Bruton, and J. E. Suarez.** 1981. Restriction mapping of the DNA of the *Streptomyces* temperate phage φC31 and its derivatives. Gene **14**:183–194.
7. **Chater, K. F., C. J. Bruton, J. E. Suarez, and W. Springer.** 1981. *Streptomyces* phages and their applications in deoxyribonucleic acid cloning, p. 380–383. *In* D. Schlessinger (ed.), Microbiology—1981. American Society for Microbiology, Washington, D.C.
8. **Chater, K. F., D. A. Hopwood, T. Kieser, and C. J. Thompson.** 1982. Gene cloning in *Streptomyces*. Curr. Top. Microbiol. Immunol. **96**:69–95.
9. **Chater, K. F., and L. C. Wilde.** 1976. Restriction of a bacteriophage of *Streptomyces albus* G involving endonuclease *Sal*I. J. Bacteriol. **128**:644–650.
10. **Clewell, D. B., and D. R. Helinski.** 1970. Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strain specificity of the relaxation event. Biochemistry **9**:4428–4440.
11. **Fraley, R., R. H. Staubinger, G. Rule, E. L. Springer, and D. Papahadjopoulos.** 1981. Liposome-mediated delivery of deoxyribonucleic acid to cells: enhanced efficiency of delivery related to lipid composition and incubation conditions. Biochemistry **20**:6978–6987.
12. **Fraley, R., S. Subramani, P. Berg, and D. Papahadjopoulos.** 1980. Introduction of liposome-encapsulated SV40 DNA into cells. J. Biol. Chem. **255**:10431–10435.
13. **Fraley, R. T., Ch. S. Fornari, and S. Kaplan.** 1979. Entrapment of a bacterial plasmid in phospholipid vesicles: potential for gene transfer. Proc. Natl. Acad. Sci. U.S.A. **76**:3348–3352.
14. **Giles, K. L., V. Vaughan, J. P. Ranch, and J. Emery.** 1980. Liposome-mediated uptake of chloroplasts by plant protoplasts. In Vitro **16**:581–584.
15. **Gregoriadis, G.** 1980. The liposome drug-carrier concept: its development and future, p. 25–86. *In* G. Gregoriadis and A. C. Allison (ed.), Liposomes in biological systems. John Wiley & Sons Ltd., Chichester, England.
16. **Hopwood, D. A.** 1967. Genetic analysis and genome structure in *Streptomyces coelicolor*. Bacteriol. Rev. **31**:373–403.
17. **Hopwood, D. A.** 1981. Genetic studies with bacterial protoplasts. Annu. Rev. Microbiol. **35**:237–272.
18. **Hopwood, D. A., and H. M. Wright.** 1978. Bacterial protoplast fusion: recombination in fused protoplasts of *Streptomyces coelicolor*. Mol. Gen. Genet. **162**:307–317.
19. **Hopwood, D. A., and H. M. Wright.** 1979. Factors affecting recombinant frequency in protoplast fusions of *Streptomyces coelicolor*. J. Gen. Microbiol. **111**:137–143.
20. **Isogai, T., H. Takahashi, and H. Saito.** 1980. High frequency protoplast transfection of *Streptomyces parvulus* 2297 with actinophage R4 DNA. Agric. Biol. Chem. **44**:2425–2428.
21. **Krügel, H., G. Fiedler, and D. Noack.** 1980. Transfection of protoplasts from *S. lividans* 66 with actinophage SH10 DNA. Mol. Gen. Genet. **177**:297–300.
22. **Lomovskaya, N. D., N. M. Mkrtumian, N. L. Gostimskaya, and V. N. Danilenko.** 1972. Characterisation of temperate actinophage φC31 isolated from *Streptomyces coelicolor* A3(2). J. Virol. **9**:258–262.
23. **Lurquin, P. F.** 1979. Entrapment of plasmid DNA by liposomes and their interactions with plant protoplasts. Nucleic Acids Res. **6**:3773–3784.
24. **Lurquin, P. F.** 1981. Binding of plasmid loaded liposomes to plant protoplasts: validity of biochemical methods to evaluate the transfer of exogenous DNA. Plant Sci. Lett. **21**:31–40.
25. **Makins, J. F., and G. Holt.** 1981. Liposome-mediated transformation of streptomycetes by chromosomal DNA. Nature (London) **293**:671–673.
26. **Mathews, B., S. Dray, J. Widholm, and M. Ostro.** 1979. Liposome-mediated transfer of bacterial RNA into carrot protoplasts. Planta **145**:37–44.
27. **Mathews, B. F., and D. E. Cress.** 1981. Liposome-mediated delivery of DNA to carrot protoplasts. Planta **153**:90–94.
28. **Mukherjee, A. B., S. Orloff, J. De B. Butler, T. Triche, P. Lalley, and J. D. Schulman.** 1978. Entrapment of metaphase chromosomes into phospholipid vesicles (lipochromosomes): carrier potential in gene transfer. Proc. Natl. Acad. Sci. U.S.A. **75**:1361–1365.
29. **Papahadjopoulos, D.** 1978. Liposomes and their uses in biology and medicine. Ann. N.Y. Acad. Sci. **308**:1–462.
30. **Papahadjopoulos, D., G. Poste, and B. E. Schaeffer.** 1973. Fusion of mammalian cells by unilamellar lipid vesicles: influence of lipid surface charge, fluidity and cholesterol. Biochim. Biophys. Acta **323**:23–42.
31. **Rollo, F., M. G. Galli, and B. Parici.** 1981. Liposome-mediated transfer of DNA to carrot protoplasts: a biochemical and autoradiographic analysis. Plant Sci. Lett. **20**:347–354.
32. **Suarez, J. E., and K. F. Chater.** 1980. Polyethylene glycol-assisted transfection of *Streptomyces* protoplasts. J. Bacteriol. **142**:8–14.
33. **Suarez, J. E., and K. F. Chater.** 1980. DNA cloning in *Streptomyces*: a bifunctional replicon comprising pBR322 inserted into a *Streptomyces* phage. Nature (London) **286**:527–529.
34. **Suarez, J. E., and K. F. Chater.** 1981. Development of a DNA cloning system in *Streptomyces* using phage vectors. Cienc. Biol. (Portugal) **6**:99–110.
35. **Szoka, F., Jr., and D. Papahadjopoulos.** 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. U.S.A. **75**:4194–4198.
36. **Thompson, C. J., J. M. Ward, and D. A. Hopwood.** 1980. DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. Nature (London) **286**:525–527.

37. **Wilson, T., D. Papahadjopoulos, and R. Taber.** 1977. Biological properties of poliovirus encapsulated in lipid vesicles: antibody resistance and infectivity in virus-resistant cells. Proc. Natl. Acad. Sci. U.S.A. **74**:3471–3475. Gene **10**:87–94.
38. **Wong, T. K., C. Nicolau, and P. H. Hofschneider.** 1980. Appearance of β-lactamase activity in animal cells upon liposome-mediated gene transfer.