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Interspecific protoplast fusion and liposome-mediated transformation in *Streptomyces*

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ABSTRACT Genetic recombination through protoplast fusion induced by polyethylene glycol (PEG) 1000 was investigated between various species of *Streptomyces*. The common parent *Streptomyces coelicolor* A3(2) strain 2709 (*pro*A1 *his*A1 *arg*A1 *cys*D18 *ura*A1 *str*A1), was fused with various strains of *S. lividans*, *S. parvulus*, *S. albus* G, *S. violaceolatus* and *S. violaceoruber* which were prototrophic and streptomycin sensitive. The fusions between *S. coelicolor* and the following species: *S. parvulus*, *S. violaceoruber* (2915) and *S. violaceolatus* gave clear negative results, while the fusions between *S. coelicolor* and *S. lividans* 66 strains 1326 and 2896 gave apparent recombination frequencies which ranged from 2.5×10^{-8} to 1.0×10^{-6}. The reversion frequencies of the auxotrophic markers in the 2709 parent was about 1.0×10^{-7}. Chromosomal marker recombination frequencies in an intraspecific protoplast fusion between strains 2709 and M145 (*S. coelicolor*), both being SCP1$^-$ SCP2$^-$, was 4.4×10^{-2} to 9.3×10^{-2}. The recombinants selected were all streptomycin resistant.

Interspecific liposome-mediated transformations of *S. coelicolor* 2709 protoplasts by donor DNA from *S. lividans* 66 strains 1326 and 2896 indicated a 10 to 100-fold increase in the recombinant frequencies over that of ordinary protoplast fusions. The frequencies obtained during transformation with *S. lividans* 1326 DNA for the recombinants *pro*$^+$ *str*^{r}, *his*$^+$ *str*^{r}, *arg*$^+$ *str*^{r}, *cys*$^+$ *str*^{r} and *ura*$^+$ *str*^{r} were 1.0×10^{-6} to 1.0×10^{-5}, while those obtained with *S. lividans* 2896 DNA were 1.2×10^{-5} to 3.5×10^{-5}.

ABSTRAK Rekombinasi genetik melalui pencantuman protoplas yang diinduskan oleh polietilene glikol (PEG) 1000 dikaji antara berbagai spesis *Streptomyces*. Induk umum *Streptomyces coelicolor* A 3(2) strain 2709 (*pro*A1 *his*A1 *arg*A1 *cys*D18 *ura*A1 *str*A1), dicantumkan dengan berbagai strain-strain spesis *S. lividans*, *S. parvulus*, *S. albus*G, *S. violaceolatus* dan *S. violaceoruber* yang jenis liar. Pencantuman di antara *S. coelicolor* dan spesis berikut: *S. parvulus*, *S. violaceoruber* (2915) dan *S. violaceolatus* memberi keputusan yang negatif manakala pencantuman di antara *S. coelicolor* dan *S. lividans* 66 strain-strain 1326 dan 2896 memberi frekuensi-frekuensi rekombinasi dari 2.5×10^{-8} hingga 1.0×10^{-6}. Frekuensi berbalik penanda-penanda auksotrofik dalam induk 2709 ialah 1.0×10^{-7}. Frekuensi rekombinasi penanda kromosom dalam pencantuman protoplas intraspesifik di antara strain-strain 2709 dan M145 (*S. coelicolor*) ialah 4.4×10^{-2} hingga 9.3×10^{-2}. Rekombinan yang dipilih ialah merintang kepada streptomycin. Transformasi antara spesis dengan menggunakan liposom (protoplas-protoplas *S. coelicolor* 2709 dengan DNA penderma dari strain-strain *S. lividans* 66 1326 dan 2896) menunjukkan penambahan 10 hingga 100 kali dalam frekuensi rekombinasi jika dibanding dengan pencantuman protoplas biasa. Frekuensi-frekuensi yang di dapati dalam transformasi dengan DNA *S. lividans* 1326 untuk rekombinan-rekombinan *pro*$^+$ *str*^{r}, *his*$^+$ *str*^{r}, *arg*$^+$ *str*^{r}, *cys*$^+$ *str*^{r} dan *ura*$^+$ *str*^{r} ialah 1.0×10^{-6} hingga 1.0×10^{-5} manakala dengan DNA *S. lividans* 2896, frekuensi sebanyak 1.2×10^{-5} hingga 3.5×10^{-5} di dapati.

(*Streptomyces*; protoplast fusion, liposomes, genetic recombination; hybrids)

INTRODUCTION

Streptomycetes are Gram-positive mycelial bacteria, members of which produce antibiotics of diverse chemical structure and mode of action. Conjugation which was the first successful technique to effect genetic recombination in *Streptomyces* [1] resulted in often very low or undetectable recombinant frequencies in some strains. Interspecific recombination through protoplast fusion holds considerable interest, particularly for industrial purposes, for example, in attempting the construction of strains making hybrid antibiotics [2].

The basic techniques of protoplast formation and regeneration were largely developed by Okanishi, Suzuki and Umezawa [3]. The fusion of protoplasts of two strains is promoted by treatment with polyethylene glycol (PEG), and in the resulting hybrid cell the DNA of the parents may be recombined. The hybrid protoplasts can then be induced to regenerate their cell wall to yield a normal culture.

Liposome-mediated transformation was a new technique developed as a means of genetic recombination in *Streptomyces* [4]. Liposomes are artificial phospholipid vesicles which can entrap DNA molecules. The original liposome preparations of Bangham *et al.* [5] which were made by mechanical shaking, consisted of multilamellar vesicles (MLV). Compounds encapsulated in liposomes are protected against enzymatic degradation and to some extent buffered against changes in pH. Rodicio and Chater [6] reported that transfection

of *Streptomyces* protoplasts by ϕ c31 DNA was stimulated by using positively charged liposome supernatants and high PEG concentration.

This paper describes the results of a study undertaken to investigate the factors involved in interspecific protoplast fusion. The hypothesis was that genetic recombination through protoplast fusion could be affected by imperfect homology of the two parental chromosomes, the possible physiological incompatibility arising from the fusion of strains making different antibiotics to which the other parent may well be sensitive and the physiological state and cytoplasmic interactions of the protoplasts. Liposome-mediated transformation was used to eliminate the physiological and cytoplasmic factors that are often apparent when two protoplasts are fused.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study were obtained from the John Innes Institute (JII), Norwich, United Kingdom as shown in Table 1.

Table 1. List of *Streptomyces* strains used in protoplast fusion.

Strain	JII stock no.	Genetic markers and plasmid status
S. coelicolor A3(2)	M145	SCP1⁻ SCP2⁻
S. coelicolor A3(2)	2709	*pro*A1 *his*A1 *arg*A1 *cys*D1 *ura*A1 *str*A1 SCP1⁻ SCP2⁻
S. parvulus ATCC 12434	2266	
S. lividans 66	1326	
S. lividans ISP 5434	2896	
S. violaceolatus ISP 5438	2910	
S. violaceoruber	2915	
S. albus G		

Media

The following media and buffers were used in this study: Yeast Extract Malt Extract Medium (YEME) [7], R2 Yeast Extract (R2YE) regeneration medium [8], Minimal medium [9], P medium [10].

In the case of auxotrophs, the YEME medium was supplemented with L-arginine, L-cystine, L-histidine, L-proline to a final concentration of 45 µg/mL and uracil to a final concentration of 9 µg/mL. The minimal medium was supplemented with these amino acids to a final concentration of 56.25 µg/mL and uracil to a final concentration of 11.25 µg/mL. The final concentration of streptomycin in the medium was 16 µg/mL.

The G-Buffer which was prepared according to Makins and Holt [4] had the following composition:- NaCl 0.015 M, trisodium citrate 0.015 M, sucrose 0.28 M, $CaCl_2$ 0.1 M, threonine 0.1 M and histidine 0.1M. The pH was adjusted to 7.0.

The liposome sedimentation buffer (LSB) consisted of 0.7 M KCl containing 10% ethanol.

Culture conditions

Spores of the various *Streptomyces* strains were inoculated into YEME medium with or without supplements in 250 mL. flasks containing coiled springs for dispersion. The cultures were incubated at 28°C for 36 to 44 hours under shaking conditions at 150 rpm.

Protoplast preparation and fusion

The standard conditions as described by Hopwood and Wright [10] were used with the following modifications. Two day old mycelia were harvested and washed twice with 20 mL of 0.3 M sucrose. The mycelium was suspended in 2.0 mL of lytic mixture and incubated at 32°C for 60 minutes to 1.5 hours depending on the strain used. The lytic mixture was prepared by dissolving 20 mg of lysozyme in 20 mL of P medium. Four ml of P medium was added and the resulting mixture was pipetted several times and filtered through a small plug of cotton wool. The protoplast titre was estimated with a haemocytometer. The protoplasts were pelleted by centrifugation and resuspended in 4 mL of medium P. This step was repeated and the protoplasts were finally resuspended in 0.5 mL of P medium.

Protoplasts of the two parent strains were fused in a balanced proportion by mixing 0.5 mL of a suspension of the respective strains. It was centrifuged for 5 minutes and the pellet was dispersed in the remaining fluid. 0.8 mL of 50% PEG was added. The protoplasts were coated with PEG by pipetting up and down with a sterile pasteur pipette once or twice and left to stand for 2 minutes. Four mL of complete P medium was added and centrifuged for 5 minutes at *ca.* 1,000 g. The supernatant was poured off and the protoplast pellet was resuspended in remaining liquid. Then 0.5 ml of complete P medium was added to the protoplasts and 0.1 mL of the suspension was plated on to the regeneration medium supplemented with growth factors required by the parents. Dilutions of protoplast suspen-

sions were made in P medium. Dilutions were plated at 10^{-1} and 10^{-2} for each parent and fusion.

Genetic characterization of recombinants

Spores were harvested from well-sporulating regeneration media plates. The spore suspension was agitated briefly on a whirlimixer, filtered through non-absorbent cotton wool and centrifuged at 1000 g for 10 minutes. The spore pellet was resuspended in 2 mL of sterile distilled water and serial dilutions were made. Dilutions at 10^{-4} and 10^{-5} were plated on parental media: Minimal medium (MM) and minimal medium + PrHACU + Str (P = proline, H = histidine, A = arginine, C = cystine, U = uracil and Str = streptomycin). The original spore suspension and dilution at 10^{-1} were plated on the selective media. These comprise of minimal media containing various combinations of the supplements (a particular supplement being omitted in each media) and streptomycin. Colonies from selective media plates were retested. These recombinants were then replicated to diagnostic media to check for segregation of other markers.

Extraction of chromosomal DNA

The procedure described by Chater *et al.* [8] was followed. The mycelium from a 48 hour culture was suspended in 25% sucrose, 0.05 M Tris pH 8. Lysozyme (10 mg/mL) was added to the final solution and incubated at 30°C. After addition of 0.5 M EDTA followed by pronase, sodium dodecyl sulphate, (3.3 %) was added and the solution was incubated at 37°C for 2 hours. The resulting mixture was phenol-chloroform extracted. The aqueous phase was removed. RNase was added to 40 µg/mL and incubated for 1 hour at 37°C. 0.2 volumes of 5M NaCl were added, the resulting solution was mixed gently and 30% PEG 6000 was added to 10% final concentration. After overnight precipitation, the suspension was centrifuged gently and the supernatant was discarded. The precipitate was dissolved in 5 ml of sterile TE containing 0.1 M NaCl. The DNA was precipitated with 0.3 M sodium acetate and 2.2 volumes ethanol. After precipitation in the cold, the DNA was sedimented by centrifugation and the pellet was washed with ethanol and dissolved in sterile TE (TE: 10 mM TrisHCl, 1 mM EDTA, pH = 8.0).

Preparation of liposomes

Neutral liposomes were prepared from L-α-phosphatidyl choline (lecithin) according to the hand-shaken method of Bangham *et al.* [5]. 14.5 mg of lecithin was dissolved in 2 mL of chloroform. This was divided into two 1 ml aliquots contained in a round bottomed flask. Nine ml of chloroform was added to each flask. This was rotary evaporated at 55°C to form a lipid film. To one of the flasks was added 0.5 mL of G buffer and 20 µg of DNA. To the other flask, only 0.5 mL of G buffer was added. The flask was hand-shaken and rotary evaporated at least 5 minutes to give the liposome-DNA suspension. To this was added 5.0 mL of liposome sedimentation buffer. The suspension was transferred to a tissue culture tube and centrifuged at 6000 rpm for 15 minutes. The liposome pellet was redissolved in the minimal amount of residual liquid and 0.5 mL of liposome sedimentation buffer was added. The supernatant and the pellet were both divided into two portions.

Liposome-mediated transformation of protoplasts

100 µL of the liposome DNA pellet (portion 1) was added to the *Streptomyces* protoplasts and fused in the presence of 50% PEG 1000. 100 µL of the liposome DNA pellet was incubated with 8 µL of DNase I at 37°C for 45 minutes. This was transferred to a tissue culture tube containing 1.0 mL of *Streptomyces* protoplasts and fusion was carried out in the presence of PEG 1000. 100 µL of the supernatant was added to the protoplasts which was to be transformed, in the presence of PEG 1000. The other portion of the supernatant (100 µL) was treated with DNase I and fused with protoplasts in the usual manner. The supernatant, which was obtained from the flask in which DNA was not added, was divided into three 100 µL aliquots. 2 µg of DNA was added to the first two aliquots and the mixture was fused with the protoplasts in the first without DNase treatment and with DNase treatment in the second. 100 µL of the third aliquot was fused with the protoplasts. Suitable dilutions were made in P medium (10^{-1}, 10^{-2}). 0.1 mL of the original suspension as well as the dilutions were plated on R2YE medium.

RESULTS

The conditions for efficient fusion and regeneration of protoplasts of the various species were established. The *S. coelicolor* and *S. lividans* strains gave good yields of protoplasts (1×10^9 to 1×10^{10} protoplasts/mL) however the viability or reversion rate of the protoplasts showed a 100 fold less titre. The optimal glycine

Table 2. Recombinant frequencies obtained in the various protoplast fusion.

Fusion	Viable count[a] (per mL)	Recombinant colonies and frequencies				
		pro$^+$ *str*^{r}	*his*$^+$ *str*^{r}	*arg*$^+$ *str*^{r}	*cys*$^+$ *str*^{r}	*ura*$^+$ *str*^{r}
2709 x M145 (intrastrain control)	3.0×10^8	280[b] 9.3×10^{-2}[c]	268[b] 8.9×10^{-2}[c]	211[b] 7.0×10^{-2}[c]	276[b] 9.2×10^{-2}[c]	132[b] 4.4×10^{-2}[c]
2709 x 2266	2.3×10^7	0	0	0	Leaky	0
2709 x 1326	3.9×10^8 2.5×10^{-8}[c]	0 4.9×10^{-7}[c]	1[d] 3.4×10^{-7}[c]	19[d]	3[d]	Leaky
2709 x 2896	2.4×10^8	1[d] 1.2×10^{-7}[c]	Leaky	2[d] 8.3×10^{-8}[c]	49[d] 2.0×10^{-6}[c]	Leak
2709 x *S. albus* G	2.1×10^7	2[d]	0 1.0×10^{-7}[c]	8[d]	0 4.0×10^{-7}[c]	0
2709 x 2910	1.1×10^7	1[d] 1.0×10^{-6}[c]	Leaky	0	Leaky	Leaky
2709 x 2915	3.8×10^7	0	0	0	0	0

[a]The viable count was obtained by plating out spores harvested from well-sporulating plates on to R2YE plates at 10^{-5} dilution.
[b]Number of colonies appearing on the selective media plates when 0.1 ml of a dilution of 10^{-3} of the spore suspension harvested from the regenerated fusion mixture was plated.
[c]Expressed as fraction of total viable spores.
[d]Number of colonies appearing on the selective media plates when 0.1 ml of the original spore suspension harvested from the regenerated plates was plated on selective media.

Table 3. Frequency of recombinants in liposome-mediated transformation of *Streptomyces lividans* strain 1326 DNA.

Selective media	MM+HACU +Str (*pro*$^+$ *str*^{r})[a]	MM+PrACU +Str (*his*$^+$ *str*^{r})[a]	MM+PrHCU +Str (*arg*$^+$ *str*^{r})[a]	MM+PrHAU +Str (*cys*$^+$ *str*^{r})[a]	MM+PrHAC +Str (*ura*$^+$ *str*^{r})[a]
Number of colonies	145	225	280	23	28
Frequencies of transformants[b]	5.8×10^{-6}	9.0×10^{-6}	1.12×10^{-5}	9.2×10^{-7}	1.12×10^{-6}

[a]Genotype scored for in the transformation
[b]The recombinant frequency was calculated as the number of recombinants per recipient parent (strain no 2709). The viable protoplast titre of 2709 was 2-3 x 10^7 /ml.

concentration for mycelial growth prior to protoplasting was found to be 0.5% for most of the *Streptomyces* strains studied.

The *S. coelicolor* strain 2709 (*pro*A1 *his*A1 *arg*A1 *cys*D18 *ura*A1 *str*A1 (SCP1$^-$ SCP2$^-$) was used as the common parent in the various interspecific protoplast fusions. The intraspecific control involved the fusion between strain 2709 and *S. coelicolor* strain M145 (SCP1$^-$ SCP2$^-$) which was wild type and streptomycin sensitive (Fig. 1). Table 2 shows the frequencies of the various recombinants scored for in the respective protoplast fusions.

The results show intraspecific recombination frequencies of 4.4 - 9.3 x 10^{-2} while interspecific recombination frequencies were as low as 2.5 x 10^{-8} to 1 x 10^{-6}. The reversion frequencies of the auxotrophic markers for the 2709 parent was 1 x 10^{-7}. *S. parvulus* and *S. violacoeruber* 2915 gave clear negative results when fused with *S. coelicolor*. Some of the recombinants were classified as leaky since the growth of these colonies was not as good as that of the wild-type on the selective media.

Chromosomal DNA from *S. lividans* 1326 (wild type) was used to transform *S. coelicolor* 2709 proto-

plasts using liposomes. Table 3 shows the frequency of recombinants in liposome-mediated transformations. Results show that genetically characterized transformants were obtained for all the markers studied with a frequency of 1.0 x 10^{-6} to 1.0 x 10^{-5}.

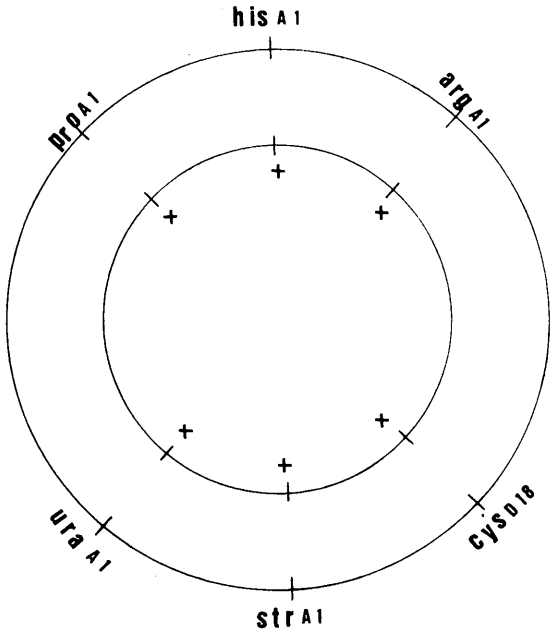

Figure 1. The genetic maps of the *S. coelicolor* chromosomes with respect to the markers studied. The outer circle represents strain 2709 and the inner circle represents strain M145.
pro = proline requiring, his = histidine requiring, arg = arginine requiring, cys = cystine requiring, ura = uracil requiring and str = streptomycin resistant
+ = wild type allele prototrophic in the case of the auxotrophic markers and streptomycin sensitive with respect to the *str* locus.

Chromosomal DNA from *S. lividans* 2896 was used to transform *S. coelicolor* protoplasts in liposome-mediated transformations, as described in the methods. The results are tabulated in Table 4. Recombinants were obtained when the liposome-DNA pellet was used to transform *S. coelicolor* protoplasts both in the presence and absence of DNase I (Deoxyribonuclease). However, no recombinants were obtained when the supernatant (which was obtained after the sedimentation of the liposome DNA pellet) was treated with DNase I prior to transformation of the protoplasts. The recovery of recombinants using the supernatant to transform the protoplasts indicated that there was some free DNA (which had not been entrapped in the liposomes) which brought about the transformation.

When the DNA-free liposome suspension was sedimented and the supernatant was used to transform the protoplasts there were no recombinants. However, when 2 µg of DNA was added to the supernatant prior to transformation, genetically characterized transformants were obtained. This activity was eliminated in the presence of DNase I. The recombinant frequencies obtained in all the cases mentioned above were 1.2 x 10^{-5} to 3.5 x 10^{-5}.

DISCUSSION

Interspecific protoplast fusion was investigated as a more efficient system of producing genetic recombinants where conventional mating or conjugation experiments were not very successful. When intraspecific protoplast fusion was carried out between *S. coelicolor* A3 (2) strains (*pro*A1 *his*A1 *arg*A1 *cys*D18 *ura*A1

Table 4. Frequency of chromosomal marker recombinants in the liposome-mediated transformation of *S. coelicolor* protoplasts by *S. lividans* strain 2896 DNA.

Transformation mixture	Recombinant genotypes				
	pro$^+$ *str*^{r}	*his*$^+$ *str*^{r}	*arg*$^+$ *str*^{r}	*cys*$^+$ *str*^{r}	*ura*$^+$ *str*^{r}
Liposome-DNA Pellet	1.6 x 10^{-5}	1.2 x 10^{-5}	2.0 x 10^{-5}	2.4 x 10^{-5}	2.3 x 10^{-5}
Liposome-DNA Pellet + DNase	4.4 x 10^{-5}	2.8 x 10^{-5}	1.5 x 10^{-5}	1.5 x 10^{-5}	1.9 x 10^{-5}
Liposome-DNA Supernatant	1.4 x 10^{-5}	2.8 x 10^{-5}	3.0 x 10^{-5}	3.5 x 10^{-5}	2.1 x 10
Liposome-DNA Supernatant + DNase	NO RECOMBINANTS PER 10^{6-7} PROTOPLASTS				
DNA-free Liposome Supernatant + 2 µg DNA	1.4 x 10^{-5}	2.8 x 10^{-5}	3.0 x 10^{-5}	3.5 x 10^{-5}	2.1 x 10^{-5}
DNA-free Liposome Supernatant + 2 µg DNA + DNase	NO RECOMBINANTS PER 10^{6-7} PROTOPLASTS				
DNA-free Liposome Supernatant	NO RECOMBINANTS PER 10^{6-7} PROTOPLASTS				

*str*A1) and M145 (prototrophic and streptomycin sensitive), both strains being SCP1$^-$ SCP2$^-$, the frequency of the five classes of recombinants (*pro*$^+$ *str*^{r}, *his*$^+$ *str*^{r}, *arg*$^+$ *str*^{r}, *cys*$^+$ *str*^{r} and *ura*$^+$ *str*^{r}) ranged from 4.4 - 9.0%. Interspecific protoplast fusions resulted in recombinant frequencies of 2.5 x 10^{-8} to 1.0 x 10^{-6} in the fusions between *S. coelicolor* and *S. lividans* strains. Clear negative results were obtained in the fusions with other species. This could be explained by poor genome homology between the strains fused. Godfrey *et al.* [11] reported values of 2.5 x 10^{-6} to 1.7 x 10^{-5} in protoplast fusions between *S. bikiniensis* and *S. fradiae*. Ochi [12] found that recombinants between *S. parvulus* and *S. antibioticus* occurred with frequencies of 3.7 x 10^{-7} to 2.2 x 10^{-6}. Robinson *et al.* [13] found high frequency of recombinants which varied from 1-9% in an interspecific protoplast fusion between *S. jumonjinensis* and *S. lipmanii*.

Makins and Holt (4) reported that *S. coelicolor* chromosomal DNA when entrappped in liposomes could transform the auxotrophic markers of the recipient to prototrophy with a frequency of up to 10%. The examples studied in their experiment were intraspecific liposome-mediated transformations. In this study, interspecific liposome-mediated transformation of *S. coelicolor* protoplasts by *S. lividans* 1326 DNA resulted in recombinant frequencies for all the markers studied of 1.0 x 10^{-6} to 1.0 x 10^{-5}.

Neutral liposomes were used to entrap DNA from *S. lividans* 2896 in the transformation of *S. coelicolor* protoplasts. The frequency of genetically characterized transformants was 1.2 x 10^{-5} to 3.5 x 10^{-5} which is about 10-100 fold that obtained in ordinary protoplast fusions. When the liposome-DNA pellet was used for transformation, recombinants were obtained irrespective of DNase I treatment. This indicates the advantage of liposome-mediated transformations over ordinary transformations where the DNA is not protected from DNase I. The supernatant which was obtained after sedimenting the liposome-DNA suspension contained free DNA which brought about transformation. This activity was eliminated when the supernatant was treated with DNase. When naked DNA (2 µg) was added to the DNA free liposome supernatant, transformants were obtained with the same frequency as that of the earlier cases. This activity was eliminated when the supernatant was treated with DNase prior to transformation. Recombinants were not obtained when the DNA free liposome supernatant was used to transform *S. coelicolor* protoplasts. This confirms that it is the DNA either in the entrapped form or in the naked state which is responsible for the transformation.

Rodicio and Chater [6] reported that there was stimulation of transfection of *S. lividans* protoplasts using positively charged liposome supernatants. In our results no such stimulation was observed, using neutral liposomes. This effect, if any, should be confirmed using positively and negatively charged liposomes.

Liposome-mediated transformations have an advantage over ordinary protoplast fusions in that the cytoplasmic interactions which are evident when two protoplasts fuse would be eliminated.

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