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Efficient Plasmid Transformation of *Streptomyces ambofaciens* and *Streptomyces fradiae* Protoplasts

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A procedure for efficient transformation of *Streptomyces ambofaciens* and *Streptomyces fradiae* protoplasts with plasmid DNA was developed. Transformation frequencies with *S. fradiae* protoplasts were strongly influenced by the temperatures for cell growth, protoplast formation, and protoplast regeneration. Transformation frequencies for both species were also influenced by the culture age before protoplast formation, the source and concentration of polyethylene glycol, the transformation-inducing agent, the concentration of protoplasts used in the transformation procedure, and the number of protoplasts added to regeneration plates. Transformation frequencies were substantially higher for both species when calf thymus DNA and protamine sulfate were added to the transformation mix. With *S. fradiae*, transformation frequencies were much lower with plasmid DNA prepared from other species than with the same plasmids prepared from *S. fradiae*, suggesting that *S. fradiae* expresses restriction and modification. With the modified transformation procedures using DNA prepared from homologous hosts, *S. ambofaciens* and *S. fradiae* are now transformed routinely at frequencies of 10^6 to 10^7 transformants per μg of plasmid DNA.

Polyethylene glycol (PEG)-induced plasmid transformation of *Streptomyces* protoplasts (13, 40) has allowed rapid development of efficient gene cloning in several *Streptomyces* spp., particularly in *Streptomyces lividans* (10–12, 15–17, 20–32, 35, 37–39). However, the transformation procedures developed primarily for *S. lividans* (13, 40) have not given high enough transformation frequencies to permit efficient gene cloning in *Streptomyces ambofaciens* and *Streptomyces fradiae*, the producers of the macrolide antibiotics spiramycin (33) and tylosin (3, 7, 8, 36), respectively. *S. ambofaciens* is a potentially useful host for gene cloning since it is relatively nonrestricting, unlike most *Streptomyces* spp. (18). *S. fradiae* is an attractive host for gene cloning since many mutants blocked in tylosin biosynthesis have been isolated which are suitable for homospecific and heterospecific complementation (7) and since several potential practical applications of cloning for antibiotic yield improvement and for formation of novel or hybrid antibiotic structures have been identified (3).

We therefore have examined many parameters which influence the efficiency of plasmid transformation of *S. ambofaciens and S. fradiae* and report here our results, which have led to approximately 1,000-fold improved transformation efficiencies over those obtained by the *S. lividans* procedure (13, 40). Transformation efficiencies of 2×10^6 to 5×10^6 transformants per μg of DNA in *S. fradiae* and 0.5×10^7 to 4.0×10^7 transformants per μg of DNA in *S. ambofaciens* can now be obtained by our modified procedures. A preliminary report of part of this work was presented elsewhere (6).

MATERIALS AND METHODS

Bacteria. *S. fradiae* M1 (2) and *S. ambofaciens* ATCC 15154 were used in this study.

Isolation of plasmid DNA. Plasmids pFJ105 (34) and pIJ702 (26), both of which carry the gene for thiostrepton resistance, were prepared by a procedure modified from that of Birnboim and Doly (14). Cultures containing plasmid were grown overnight in tryptic soy (TS) broth containing thiostrepton (5 μg/ml) obtained from E. R. Squibb & Sons, Inc., Princeton, N.J., and then homogenized. Homogenized mycelia (5 ml) were added to a sterile 50-ml Sorvall tube and centrifuged at 1,100 × *g* for 10 min. The supernatant was decanted, and the pellet was suspended in 5 ml of a solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris (pH 8.0), and lysozyme (5 mg/ml) obtained from Calbiochem-Behring, La Jolla, Calif. After the pellet was suspended, the mixture was placed in an ice bath for 30 min. A portion (10 ml) of a solution containing 0.2 M NaOH and 1% sodium dodecyl sulfate was then added. (Both the lysozyme and the alkaline sodium dodecyl sulfate solutions should be prepared just before use.) The contents of the tube were mixed gently until clearing was complete. The tube was placed on ice for 5 min, and then 7.5 ml of a solution containing 3.0 M sodium acetate (pH 4.8) was added and mixed by gentle inversion of the tube. The mixture was further incubated on ice for 1.0 to 1.5 h and centrifuged for 10 min at 10,000 × *g*. The clear supernatant was transferred to a sterile Sorvall tube, and the DNA was precipitated by adding 2 volumes of chilled ethanol and storing the mixture at −20°C for 2 h. The DNA was pelleted by centrifugation at 22,500 × *g* for 30 min and then resuspended in 0.5 ml of TE buffer (10 mM Tris [pH 8.0] and 1.0 mM EDTA). The DNA was transferred to a sterile 1.5-ml Eppendorf tube, and 50 μl of 3 M sodium acetate (pH 8.0) was added and mixed. Cold ethanol (1 ml) was added, and the tube was placed on dry ice for about 10 min and then centrifuged at 15,600 × *g* for 5 min. The DNA was resuspended in 0.5 ml of TE buffer, and the precipitation procedure was repeated twice. The DNA was washed with 70% ethanol and then resuspended in 100 μl of TE buffer. The plasmid DNA was purified from chromosomal DNA on a 1% agarose gel. The plasmid DNA was recovered from the gel by using a modification of the procedure of Dretzen et al. (19) developed by Paul Rostek, Eli Lilly and Co. (personal communication). After electrophoresis the gel was stained with ethidium bromide (5 μg/ml) in electrophoresis buffer for 15 min. During observation under UV light, horizontal slits were made in front of and behind the plasmid band. Strips of

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Whatman DE81 DEAE-cellulose paper cut to a height slightly greater than the thickness of the gel and a width slightly greater than the slit width were inserted into the slits. The paper strip behind the plasmid band was used to prevent contamination by DNA of higher molecular weight. The gel was squeezed firmly against the papers to close the slits and moistened with electrophoresis buffer. Electrophoresis was resumed until the DNA had entered the paper strips, which was verified by observation with UV illumination. To recover the DNA from the DEAE paper, the strips were placed in a sterile polypropylene screw cap tube. The paper was washed with 5 ml of 10 mM Tris hydrochloride (pH 8)–10.0 mM KCl and drained thoroughly. Then 5 ml of 10 mM Tris hydrochloride (pH 8)–1.0 M NaCl was added to the paper, and the mixture was swirled in a Vortex mixer until the paper was dissolved. The paper was removed by passing the mixture through a syringe containing a small amount of siliconized glass wool and into a 5-in (12.7-cm) Pasteur pipette packed loosely with a very small amount of siliconized glass wool. The eluate was collected, diluted with an equal volume of water, and precipitated with 2 volumes of ethanol. The precipitation procedure was repeated twice, and the DNA was washed with 70% ethanol. The DNA was resuspended in TE buffer, and its concentration was determined by absorbance at 260 nm.

Transformation of protoplasts. *S. fradiae* cells were grown at 29°C in TS broth plus 0.4% glycine (2, 5) to 500 to 1,000 Klett units. *S. ambofaciens* cells were grown in TS broth plus 0.4% glycine to 1,500 to 2,500 Klett units at 29 to 37°C (5). The cells were homogenized, sonicated, and washed as described previously (2, 5). The mycelia were resuspended by shaking in an equal volume of P medium (2) containing lysozyme (1 mg/ml) and placed in an ice bath for 1 h. The resulting protoplasts were washed two times with P medium, resuspended in an equal volume of P medium, and then chilled for at least 15 min. The protoplast suspension was warmed to room temperature before transformation. For *S. fradiae* and *S. ambofaciens*, transformations were carried out at room temperature as follows. Calf thymus DNA (0.8 μg; Calbiochem) was added from a stock solution (0.25 mg/ml) to the bottom of a sterile 1.5-ml Eppendorf micro test tube. Protamine sulfate (1.5 μg; Calbiochem) was added to the calf thymus DNA from a 0.1% stock solution in P medium, mixed, and incubated for 1 min at 23°C. In a separate tube, plasmid DNA in TE buffer was mixed with P medium to a final volume of 10 μl; this was then added to the calf thymus-protamine sulfate mixture. Protoplasts were diluted threefold in P medium, and 200 μl of the diluted protoplasts was added to the mixture. For *S. fradiae* transformations, 900 μl of filter-sterilized 55% PEG 1000 (usually from Fluka Chemical Corp., Hauppauge, N.Y. or Sigma Chemical Co., St. Louis, Mo.) in P medium was added to the mixture. For *S. ambofaciens* transformations, 500 μl of 55% PEG 1000 in P medium was added. The contents of the tubes were mixed gently and incubated at room temperature for 1 min. For *S. ambofaciens* transformations, an additional 400 μl of P medium was added to bring the final volume to 1 ml. For both *S. fradiae* and *S. ambofaciens* transformations, the tubes were briefly swirled in a Vortex mixer. Dilutions in P medium were made quickly, and the transformed protoplasts were plated rapidly on modified R2 agar plates in soft agar overlays (5). *S. fradiae* protoplasts were incubated at 29°C, and *S. ambofaciens* protoplasts were incubated at 34°C for 16 to 24 h; then the plates were overlaid with a second R2 soft agar overlay (5) containing thiostrepton to give a final concentration of 25 μg/ml after equilibration with the bottom agar. The plates were further incubated at the same temperatures. Thiostrepton-resistant *S. ambofaciens* transformants were counted after 3 to 4 days of incubation, and thiostrepton-resistant *S. fradiae* transformants were counted after 7 to 10 days of incubation.

In some cases, additional salt was added to the DNA before transformation. Sodium acetate was added to a 0.3 M final concentration in the DNA solution for *S. ambofaciens* transformations, and sodium chloride was added to a 0.5 M final concentration for *S. fradiae* transformations. Unless stated otherwise in the text or figure legends, transformations were performed without the addition of sodium acetate or sodium chloride to the DNA.

Protoplast regeneration. The efficiency of protoplast regeneration was determined by dividing the number of colonies derived from protoplasts by twice the number of colonies obtained from sonicated mycelia before protoplast formation as described previously (2, 5). (The number of colonies obtained from sonicated mycelia is doubled since about 50% of the potential CFUs are ruptured in the sonication process to obtain single cells [2].)

RESULTS AND DISCUSSION

Preliminary studies. Initial attempts to transform *S. fradiae* M1 with plasmid pFJ105 by the procedure of Bibb et al. (13) yielded about one transformant per microgram of plasmid DNA . The transformation frequency was increased about 10^3-fold when transformations were carried out with plasmid DNA prepared from *S. fradiae* transformants. This suggested that *S. fradiae* expresses restriction and modification and that restriction severely inhibits transformations with unmodified DNA. This is consistent with previous observations which indicate that *S. fradiae* restricts the plaque formation of several broad-host-range *Streptomyces* bacteriophage (18).

A frequency of 1,000 transformants per μg of DNA was not adequate to allow efficient homologous gene cloning in *S. fradiae*. Further, the extremely low frequencies of transformation with plasmid DNA prepared from other species clearly excluded the possibility of heterologous gene cloning to produce hybrid antibiotic structures (3). The transformation frequencies obtained with *S. ambofaciens*, a relatively nonrestricting species (18) that produces protoplasts that readily regenerate viable colonies (5), by the transformation method of Bibb et al. (13) yielded only about 10^4 transformants per μg of plasmid DNA (34).

Since the procedure of Bibb et al. (13) did not yield satisfactory levels of transformation with *S. fradiae* and *S. ambofaciens*, we initiated studies to improve the transformation efficiencies with these two species with DNA prepared from homologous hosts. Preliminary studies indicated that the efficiency of transformation of *S. fradiae* was dramatically influenced by the temperature for cell growth before protoplast formation and to a lesser extent by the temperature for protoplast formation. The best results were obtained when cells grown at 29°C were converted to protoplasts by lysozyme treatment at 4 to 15°C and when the resulting protoplasts were incubated at 29°C on modified R2 agar for cell regeneration after transformation. When the cell growth temperature was increased to 34 and 37°C, the transformation frequencies decreased about 10- and 100-fold, respectively. The transformation frequencies were less influenced by the temperature for regeneration, but gradually decreased with increasing temperature between 29 and 37°C. Previous studies had shown that the temperatures for cell growth and protoplast regeneration can dramatically influence the ef-

ficiency of cell regeneration from protoplasts of *S. fradiae* (5) and that higher temperatures were deleterious. Thus, the reduced transformation frequencies observed with protoplasts prepared from *S. fradiae* cells grown at relatively high temperatures (34 to 37°C) probably result from inefficient regeneration of transformed protoplasts.

The transformation efficiency of *S. ambofaciens* protoplasts was insensitive to variations in the temperatures for cell growth and protoplast regeneration in the 29-to-37°C range. *S. ambofaciens* protoplasts prepared and regenerated over this range of temperatures yielded very similar regeneration efficiencies in earlier studies (5).

Effects of protoplast concentration and stabilizing buffers. In the *S. lividans* procedures (13, 40), protoplasts were concentrated about 20-fold before transformation. We found that concentration of *S. fradiae* and *S. ambofaciens* protoplasts tenfold inhibited transformation by about three- to tenfold, whereas dilution of protoplasts about two- to threefold resulted in two- to threefold increases in transformation frequencies. Also, the number of protoplasts plated on modified R2 plates after transformation influenced the final frequency of transformants. Maximum transformation frequencies were obtained when about 10^4 protoplasts were added per plate. About 10-fold lower frequencies were obtained when about 3×10^5 to 1×10^6 protoplasts were added per plate (Fig. 1).

The most recent modified transformation procedure for *S. lividans* uses L medium during the lysozyme treatment to prepare protoplasts and T medium during the transformation (40). The original procedure for *S. lividans* used P medium for both steps (13). We found in transformations of *S. ambofaciens* and *S. fradiae* with plasmid pFJ105 that the use of P medium for both the lysozyme treatment and the transformation steps resulted in about two- to threefold higher frequencies of transformants than were obtained when L medium and T medium were used during these steps. The use of combinations of either P medium during lysozyme treatment and T medium during transformation or L medium during lysozyme treatment and P medium during transformation gave results equivalent to those obtained when using P medium during both steps. Since there was no clear advantage to using either L medium or T medium, but a disadvantage to using both, we routinely used P medium for both the lysozyme treatment and the transformation steps.

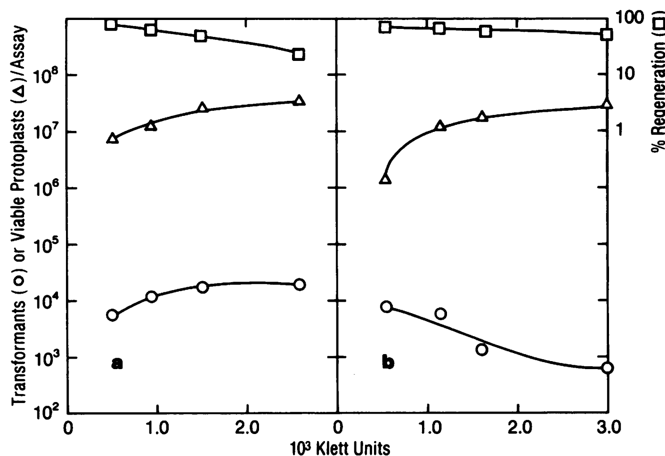

FIG. 2. Effects of cell growth phase before protoplast formation on transformation. *S. ambofaciens* (panel a) and *S. fradiae* (panel b) cells were grown in TS broth to various cell densities, and protoplasts were prepared. Protoplasts were transformed to thiostrepton resistance with plasmid pFJ105 at 7 ng per assay. Viable protoplasts per transformation assay (△) were determined by diluting protoplasts in P medium (untreated with PEG) and plating on modified R2 agar.

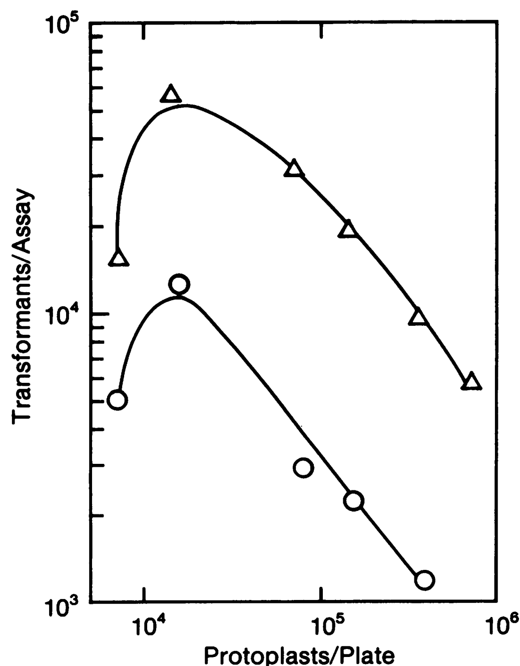

FIG. 1. Effects of protoplast concentration on transformation. Protoplasts of *S. ambofaciens* (△) and *S. fradiae* (○) were transformed to thiostrepton resistance with plasmid pFJ105 at 7 ng per assay.

Effects of cell growth phase on transformation. Figure 2a shows that with *S. ambofaciens*, the efficiency of protoplast regeneration decreased from about 80% to less than 30% of potential CFUs when cells were grown from about 500 to 2,500 Klett units, but the number of viable protoplasts increased about fourfold and the number of transformants increased about three- to fourfold over this range. The ratio of transformants to viable protoplasts present in the transformation mix before the addition of PEG was relatively constant over this cell density range (ca. 10^{-3}). Thus, the efficiency of transformation does not seem to correlate directly with the efficiency of protoplast regeneration but may be more influenced by the absolute number of viable protoplasts in the assay. In any event, protoplasts prepared from cells grown to 1,500 to 2,500 Klett units gave the best results, in this case about 2×10^4 transformants per 7 ng of DNA or about 3×10^6 transformants per μg of DNA.

The efficiency of protoplast regeneration of *S. fradiae* protoplasts decreased only moderately (from 80 to 50% maximum regeneration) as the cell density before protoplast formation was increased from 1,500 to 3,000 Klett units (Fig. 2b). The number of viable protoplasts increased about 10-fold, but the number of transformants decreased by 10-fold over this range. Thus, with *S. fradiae*, the efficiency of transformation is not totally dependent upon the number of viable protoplasts per assay or the efficiency of protoplast regeneration. It appears that the growth phase of the cells has a large influence on the efficiency of transformation. The best results are obtained when cells are grown to about 500

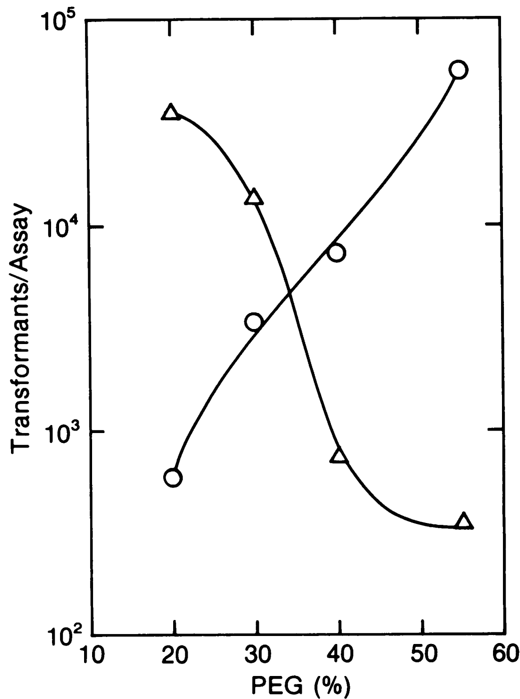

FIG. 3. Effects of source and concentration of PEG on transformation. *S. ambofaciens* protoplasts were transformed with plasmid pFJ105 at 7 ng per assay. The Fluka PEG 1000 (○) was from lot number 207858-182 and the Koch-Light PEG 1000 (△) was obtained from David Hopwood. PEG (%) indicates the concentration (wt/vol) of PEG before being mixed with protoplasts.

to 1,000 Klett units. This corresponds to the late-exponential-to-early-transition phase of growth before stationary phase (2). The efficiency of transformation per protoplast was about 0.5×10^{-3} to 5×10^{-3} with protoplasts prepared from cells in this growth phase and about 10^6 transformants per μg of DNA.

Effects of PEG on transformation. PEG induces plasmid transformation of *Streptomyces* protoplasts. In the *S. lividans* procedures (13, 40), 20% PEG induced maximum transformation. In our initial studies on transformation of *S. fradiae* and *S. ambofaciens*, we found that several different preparations of PEG induced transformation most efficiently when higher concentrations were used. A particular lot of PEG (obtained from Fluka Chemical Corp.) gave highest transformation frequencies when a 55% solution was added to the transformation mix (Fig. 3). At 20% PEG, 100-fold fewer transformants were obtained. However, Koch-Light PEG obtained from D. Hopwood, John Innes Institute, and used in the *S. lividans* transformation studies (40) gave maximum transformation when a 20% solution was added to the transformation mix (Fig. 3). Higher concentrations gave a marked inhibition (100-fold at 55% PEG).

In other experiments, we noted that most sources of PEG (including several lots from Sigma Chemical Co.) gave maximum transformation when a 55% solution was added to the transformation mix. However, some lots reproducibly gave up to 10-fold lower maximum transformation frequencies than those reported in Fig. 3. Therefore, the source and concentration of PEG are very important for efficient transformation, and it is useful to test several sources of PEG and to use only those that give high frequencies of transformants.

Enhancement of transformation by protamine sulfate and heterologous DNA. It has been shown that the addition of protamine sulfate to bacteriophage transfection or transformation mixes with *Escherichia coli* spheroplasts can cause increased frequencies of transfection and transformation (1, 4, 9). Addition of low levels of protamine sulfate to the *Streptomyces* transformation mix caused substantial enhancement of transformation frequencies with *S. ambofaciens* and *S. fradiae* protoplasts; the enhancement was even greater when calf thymus DNA was also added to the assay (Fig. 4). The maximum transformation frequencies were obtained when protamine sulfate was added at 1.5 μg per assay and calf thymus DNA was added at 0.8 μg per assay (Fig. 4). In the absence of protamine sulfate and calf thymus DNA, transformation frequencies were much lower with both species (data not shown). The order of addition of protamine sulfate, calf thymus DNA, transforming DNA, protoplasts, and PEG was also important. The highest transformation frequencies were obtained when the order of addition was as follows: calf thymus DNA, protamine sulfate, plasmid DNA, protoplasts, and then PEG. The transformation frequency was 10-fold lower when plasmid DNA was added first, followed by the addition of protamine sulfate and then calf thymus DNA, and it was fourfold lower when the addition of plasmid DNA was followed by the addition of calf thymus DNA and then protamine sulfate. It therefore appears that binding of protamine sulfate to the carrier calf thymus DNA is important for efficient transformations.

Enhancement of transformation in *Streptomyces* spp. by the addition of calf thymus DNA might be due to competitive protection against endonucleolytic cleavage of plasmid DNA before or during uptake by protoplasts. The enhancement of transformation by protamine sulfate might also be due to the inhibition of endonuclease (1) or to the formation of DNA-protamine complexes which might penetrate the protoplasts more effectively (9). However, we have no data to support either of these mechanisms.

Effects of DNA concentration and salt concentration on transformation. It was shown in a transformation procedure for *S. lividans* that the addition of certain inorganic salts caused reproducibly small increases in transformation frequencies (T. Kieser, personal communication). We have shown that the addition of 0.3 M sodium acetate to the DNA

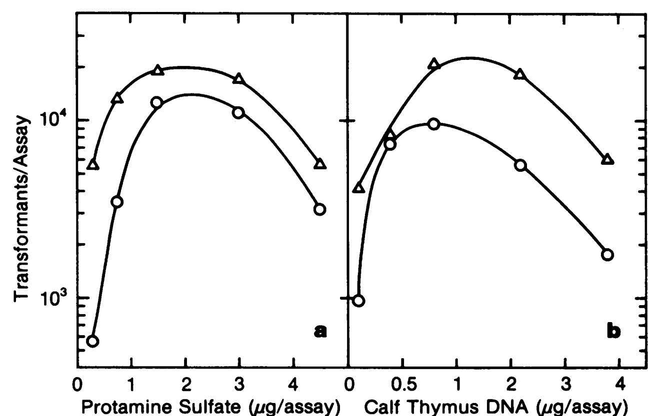

FIG. 4. Effects of protamine sulfate and calf thymus DNA on transformation. *S. ambofaciens* (△) and *S. fradiae* (○) protoplasts were transformed with plasmid pFJ105 at 7 ng per assay. In panel a, calf thymus DNA was kept constant at 0.8 μg per assay. In panel b, protamine sulfate was kept constant at 1.5 μg per assay.

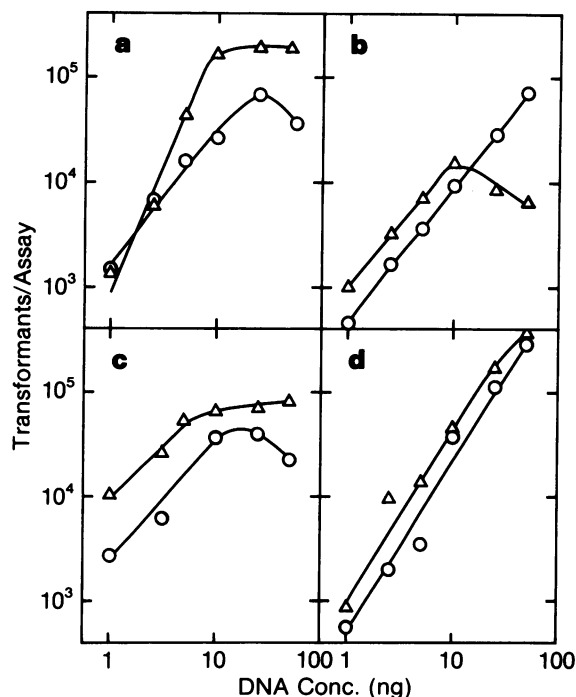

FIG. 5. Effects of DNA concentration and salt concentration on transformation. *S. ambofaciens* protoplasts were transformed with plasmid pFJ105 (panel a) and pIJ702 (panel b). Symbols (panels a and b): ○, control transformation; △, sodium acetate added to 0.3 M in the DNA solution before transformation. *S. fradiae* protoplasts were transformed with plasmids pFJ105 (panel c) and pIJ702 (panel d). Symbols (panels c and d): ○, control transformation; △, sodium chloride added to 0.5 M in the DNA solution before transformation.

solution caused increased transformation of *S. ambofaciens* with plasmids pFJ105 and pIJ702 at DNA concentrations of 10 ng or below (Fig. 5a and b). The enhancement of transformation observed with pFJ105 was due primarily to a change in the relationship between the number of transformants and the DNA concentration. When sodium acetate was added, the number of transformants increased proportionately with the square of DNA concentration (i.e., slope of 2 on a log-log plot) rather than with the first power of DNA concentration as was obtained in the control lacking sodium acetate (Fig. 5a). A slope of two suggests that a bimolecular interaction is stimulated by salt and that more efficient transformation is obtained from two DNA molecules than from a single molecule. Although this unusual relationship is highly reproducible with pFJ105, it was not observed with pIJ702 (Fig. 5b). In this case, increased salt concentration simply increased the frequency of transformants obtained at all DNA concentrations up to about 10 ng, but had no apparent effect on the slope.

With *S. fradiae* protoplasts, the addition of 0.5 M sodium chloride to the DNA solution increased the efficiency of transformation two- to threefold with plasmids pIJ702 and pFJ105 at all DNA concentrations up to about 10 ng (Fig. 5c and b). About 5×10^4 transformants were obtained from 10 ng of DNA in both cases.

The frequencies of transformants obtained with *S. ambofaciens* and *S. fradiae* are now comparable to the best frequencies obtained with *S. lividans* (40), that is, about 10^6 to 10^7 transformants per μg of DNA. We carried out transformation of *S. lividans* protoplasts using our procedure for *S. ambofaciens* and using the procedure of Thompson et al. (40), and we obtained equally high transformation frequencies with both procedures. Since our procedure uses about 50-fold fewer protoplasts, it yields a higher frequency of transformed protoplasts. At 10 ng of DNA, which often gives maximum transformation efficiency in our system, the ratio of DNA molecules to viable protoplasts is usually about 50 to 150, and the efficiency of transformation per viable protoplast ranges between 0.1 and 1.0% for both *S. ambofaciens* and *S. fradiae*. It is not known whether higher frequencies of protoplast transformation are technically feasible with PEG treatment.

The procedure described here should facilitate homologous gene cloning in both *S. ambofaciens* and *S. fradiae* and possibly heterologous gene cloning in *S. ambofaciens*, a strain that is relatively nonrestricting (18). Mutants of *S. fradiae* defective in several restriction systems have been isolated recently (P. Matsushima and R. H. Baltz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, H128, p. 112) and should be useful for heterologous gene cloning in this important species as well.

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