

Protoplast Fusion in *Streptomyces*: Conditions for Efficient Genetic Recombination and Cell Regeneration

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Protoplasts from four different species of *Streptomyces* regenerated cells efficiently in hypertonic soft agar medium overlaid on partially dehydrated regeneration medium. The efficiencies of regeneration were strongly dependent upon the incubation temperatures for cell growth and for protoplast regeneration. Cell growth temperatures (before protoplast formation) required for efficient protoplast regeneration varied from species to species, and did not necessarily correlate with the optimum temperatures for protoplast regeneration. Under the best conditions, protoplasts from all four species were able to regenerate viable cells at nearly 100% efficiency and also formed confluent lawns of mycelia when plated in high concentrations. The temperatures for cell growth and protoplast regeneration also affected the frequencies of genetic recombinants obtained by protoplast fusion in *S. fradiae*, and highest recombinant frequencies were obtained under conditions which favoured efficient protoplast regeneration. With the modified procedure described, maximum frequencies of genetic recombinants were obtained by treating parental protoplasts with 40 to 60% polyethylene glycol 1000.

INTRODUCTION

Genetic recombination in bacteria by polyethylene glycol (PEG)-induced protoplast fusion was first demonstrated in the genus *Bacillus* (Fodor & Alföldi, 1976; Schaeffer *et al.*, 1976). Subsequently, protoplast (or spheroplast) fusion techniques have been developed for *Streptomyces* (Hopwood *et al.*, 1977; Baltz, 1978, 1980; Godfrey *et al.*, 1978; Hopwood & Wright, 1978, 1979; Ochi *et al.*, 1979), *Brevibacterium* (Kaneko & Sakaguchi, 1979), *Providencia* (Coetzee *et al.*, 1979) and *Streptosporangium* (Oh *et al.*, 1980).

Protoplasts of *Streptomyces* are also readily transformed by plasmid DNA (Bibb *et al.*, 1978) and transfected by actinophage DNA (Chater, 1980; Suarez & Chater, 1980*a*), and these techniques have facilitated the development of gene cloning in this economically important genus (Bibb *et al.*, 1980*a, b*; Suarez & Chater, 1980*b*; Thompson, *et al.*, 1980).

Development of highly efficient protoplast fusion and protoplast transformation techniques in *Streptomyces* has been facilitated by the observations that protoplasts from a variety of *Streptomyces* species can regenerate viable cells on hypertonic R2 or modified R2 medium (Okanishi *et al.*, 1974; Hopwood, *et al.*, 1977; Baltz, 1978). Protoplasts from several species of *Streptomyces* are capable of regenerating confluent lawns of mycelia when plated on R2 medium at high concentrations. However, certain *Streptomyces* species produce auto-inhibitory substance(s) which inhibit regeneration of protoplasts as confluent lawns (Hopwood *et al.*, 1977; Baltz, 1978), thus making genetic analysis of recombinants tedious (Baltz, 1980) and transformation impractical.

The purpose of the present study was to identify conditions to minimize auto-inhibition of protoplast regeneration, and to maximize the efficiencies of protoplast regeneration and

protoplast fusion. We report here that protoplast regeneration and fusion efficiencies are strongly influenced by the incubation temperatures for cell growth and protoplast regeneration, and that auto-inhibition can be minimized by incubating protoplasts in soft agar overlays on partially dehydrated regeneration media. A preliminary account of part of this work has been presented elsewhere (Baltz & Matsushima, 1980).

METHODS

Streptomyces strains. The *Streptomyces* strains used in this study are listed in Table 1.

Media. TS broth contained 30 g Trypticase Soy Broth (BBL) per litre distilled water. Modified R2 medium was as described (Baltz, 1978). Hypertonic soft agar overlays (3 ml each) contained sucrose, MgCl₂, CaCl₂ and TES buffer at the concentrations used in R2 medium (Okanishi *et al.*, 1974), plus 0.65% (w/v) Bacto agar (Difco). P medium was as described (Okanishi *et al.*, 1974).

Protoplast formation. The *Streptomyces* strains were grown in TS broth and fragmented by ultrasonic treatment as described (Baltz, 1978). Ultrasonic fragments containing viable cells were added to TS broth plus glycine and grown for four to seven culture doublings as described (Baltz, 1978). The glycine concentrations required to retard growth were 0.4% (w/v) for *S. fradiae* strains, 1.2% for *S. albus* G, 0.4% for *S. ambofaciens* and 0.8% for *S. griseofuscus*. Mycelia were homogenized and sonicated for 3 s at 76 W power output as described (Baltz, 1978). Ultrasonic fragments of mycelia were diluted 1:20 into fresh TS broth plus glycine and grown to absorbances at 600 nm (A_{600}) of between 2.0 and 7.0. Mycelia were then converted to protoplasts by lysozyme-treatment in P medium as described (Baltz, 1978). A portion of each (non-lysozyme treated) culture was fragmented by ultrasound (3 s at 76 W) to obtain total viable cell counts. Under these ultrasonic treatment conditions, approximately one-half of the potential viable cells were retrieved as colony-forming units (Baltz, 1978). Therefore, colony-forming units obtained after ultrasonic treatment were doubled for protoplast regeneration efficiency calculations (see below).

Protoplast regeneration. *Streptomyces fradiae* C373 protoplasts were plated in soft agar overlays on freshly poured modified R2 plates or on modified R2 agar plates previously dehydrated by incubating plates at 23, 34 or 37 °C for various lengths of time. Unless stated otherwise in the text, modified R2 agar was routinely dehydrated 15 to 20%, and is referred to as regeneration medium. The data presented in Figs 2, 3 and 4 are from illustrative experiments, and individual data points represent single determinations.

The time course of cell regeneration (colony formation) from protoplasts was followed visually, and regeneration efficiency was estimated by dividing the number of colonies obtained by twice the number of colony-forming units obtained from ultrasonic fragmentation of mycelia before lysozyme treatment. Calculation of regeneration frequency per visible protoplast in a Petroff-Hauser counter by phase-contrast microscopy gave similar results (see Baltz, 1978), but such determinations were less accurate.

Protoplast fusion and genetic recombination. To determine the optimum polyethylene glycol (PEG) concentration to induce protoplast fusion and genetic recombination in *S. fradiae*, protoplasts of AR11 and AR60 (0.5 ml each) prepared from cells grown at 29 °C were mixed and centrifuged in a clinical centrifuge. The pellet was resuspended in 0.1 ml P medium and mixed with 0.9 ml PEG 1000 or PEG 6000 of various concentrations. After 30–60 s at 23 °C, the PEG-treated protoplasts were diluted and plated on regeneration medium and incubated at 29 °C. The percentage recombination was determined as the ratio of prototrophic colonies arising on regeneration medium to the number of colonies arising on regeneration medium supplemented with cysteine and

Table 1. *Streptomyces* strains used

Strain	Relevant characteristics	Source‡
<i>S. albus</i> G J1074	<i>ilv-1</i> , R ⁻ /M ⁻ *	Chater & Wilde (1980)
<i>S. ambofaciens</i> (ATCC 15154)	prototroph	Lilly Culture Collection
<i>S. fradiae</i> C373 (ATCC 19609)	prototroph†	Lilly Culture Collection
<i>S. fradiae</i> AR11	<i>cysDI</i>	NTG-induced (Radue & Baltz, unpublished)
<i>S. fradiae</i> AR60	<i>metA17</i>	NTG-induced (Radue & Baltz, unpublished)
<i>S. griseofuscus</i> (ATCC 23916)	prototroph	Lilly Culture Collection

* Lacks *SalI* (*SalGI*) restriction and modification.

† This *S. fradiae* strain produces the macrolide antibiotic, tylosin (Seno *et al.*, 1977).

‡ NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

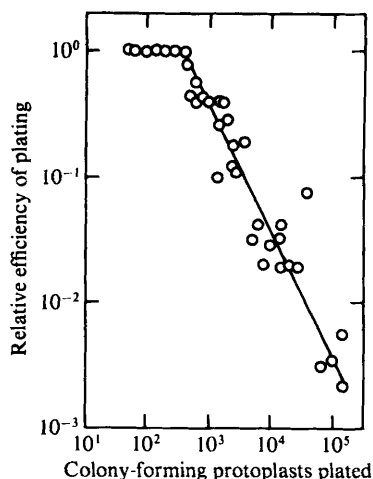


Fig. 1. Relative efficiencies of cell regeneration at different protoplast concentrations. Several different preparations of *S. fradiae* C373 protoplasts were diluted in P buffer and plated on undehydrated modified R2 agar plates by the spread-plate technique and incubated at 34 °C to regenerate viable cells (Baltz, 1978). Relative efficiencies of cell regeneration were determined by plating serial dilutions of protoplasts and normalizing the data on regeneration frequencies (per protoplast) to the maximum efficiencies obtained with highly diluted protoplasts. Each point represents a single determination.

methionine at 100 µg ml⁻¹ (Method 1) or to the sum of the colonies arising on individual plates containing regeneration medium supplemented with cysteine or methionine (Method 2). These methods are referred to as 'serial dilution techniques'. An alternative 'confluent lawn technique' (Method 3) was used in some experiments. With this technique, fused protoplasts were plated in high concentrations (> 10⁵ per plate) on regeneration medium supplemented with methionine and cysteine at 100 µg ml⁻¹. Confluent lawns of regenerated mycelia were scraped from the plates, homogenized in TS broth, grown for about 16 h at 34 °C in a water bath shaker as described (Baltz, 1978), then homogenized and sonicated (Baltz, 1978). Prototrophic recombinant frequencies were determined by plating cells on CDA (minimal) and AS-1 (complex) agar plates (Baltz, 1980).

RESULTS

Auto-inhibition of protoplast regeneration with S. fradiae

It has been noted previously with certain *Streptomyces* species that colonies which develop early from non-protoplasted cells, or from rapidly regenerating protoplasts, can inhibit the regeneration of nearby protoplasts (Hopwood *et al.*, 1977; Baltz, 1978). Figure 1 shows that with the original procedure for protoplast formation and regeneration of *S. fradiae* (Baltz, 1978) the efficiency of regeneration of cells from protoplasts decreased dramatically as the protoplast density increased beyond about 1000 protoplasts per plate. In fact this 'auto-inhibition' was so severe that no more than about 1000 colonies developed even when as many as 10⁵ protoplasts potentially capable of regenerating were spread on each plate.

Effects of dehydration of modified R2 agar on regeneration of S. fradiae protoplasts

In the initial procedure for regeneration of *S. fradiae* protoplasts (Baltz, 1978), protoplasts were spread on the surface of modified R2 medium and incubated at 34 °C. We have recently observed that auto-inhibition during regeneration can be almost completely eliminated by allowing protoplasts to regenerate in soft agar overlays plated on partially dehydrated R2 agar medium. Figure 2 shows the time course of protoplast regeneration in soft agar overlays plated on modified R2 medium which had been dehydrated to various extents. On undehydrated modified R2 agar plates, a small fraction of the protoplasts regenerated rapidly, but the remaining protoplasts capable of regeneration did not begin to form visible colonies until after 6 d incubation. Between 6 and 12 d, the number of colonies from regenerated

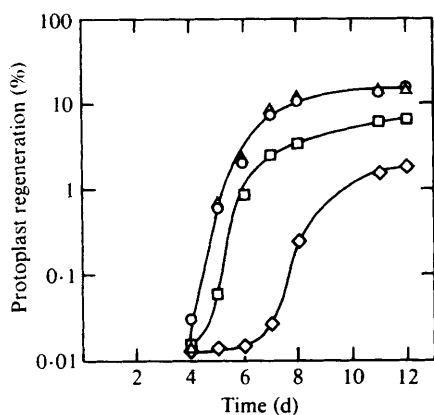


Fig. 2

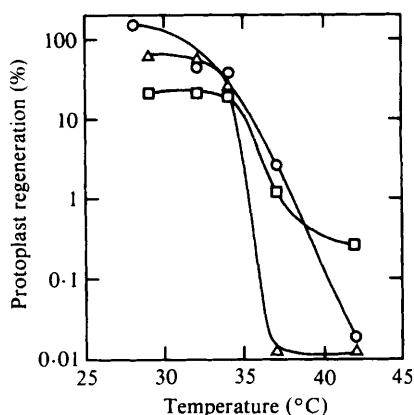


Fig. 3

Fig. 2. Regeneration of cells from protoplasts on fresh or partially dehydrated modified R2 agar plates. Cells of *S. fradiae* C373 were grown at 34 °C in TS broth plus glycine before protoplast formation. Protoplasts were prepared, diluted, added to soft agar overlays, and plated on undehydrated and dehydrated modified R2 agar plates. Colonies were counted after various times of incubation, and the percentage regeneration was determined. ◇, No dehydration; □, 5% dehydration; △, 15% dehydration; ○, 22% dehydration.

Fig. 3. Effects of temperatures for cell growth and for protoplast regeneration on the efficiency of protoplast regeneration with *S. fradiae* C373. ○, Cells grown at 29 °C and protoplasts regenerated at various temperatures; □, cells grown at various temperatures and protoplasts regenerated at 34 °C; △, cells grown and protoplasts regenerated at the same temperatures.

protoplasts increased to a maximum of less than 2% of the potential viable protoplasts. On plates containing high concentrations of protoplasts, the fraction of protoplasts which regenerated early inhibited regeneration of surrounding protoplasts, as had been noted previously (Baltz, 1978; and Fig. 1). Protoplasts plated on modified R2 agar which had lost about 5% weight by dehydration regenerated much more rapidly, and produced nearly confluent lawns when plated in high concentrations. Regeneration efficiency was about 7% (Fig. 2). Protoplasts plated on modified R2 agar which had lost 15 or 22% weight by dehydration regenerated even faster, and produced confluent lawns of mycelia when high concentrations were plated. Maximum regeneration was about 15%. Concentration of nutrients to the same extent had no effect on regeneration efficiency (data not shown).

Effects of temperature on regeneration of S. fradiae protoplasts

In previous studies on protoplast fusion with *S. fradiae* (Baltz, 1978, 1980), cells were grown and protoplasts were regenerated routinely at 34 °C. We have recently found that 34 °C is not the best temperature for efficient regeneration. Figure 3 shows a summary of results from an experiment designed to assess the effects of temperature during cell growth (before protoplast formation) and during protoplast regeneration on the efficiency of protoplast regeneration. When cells were grown at 29 °C and protoplasts from these cells were plated at different temperatures for cell regeneration, the optimum temperature for regeneration was 29 °C. At this temperature, virtually all of the potential viable protoplasts regenerated to form colonies. As the temperature for regeneration was increased, the efficiency of regeneration decreased dramatically, and at 42 °C, only about 1 in 10⁴ protoplasts were capable of regenerating viable cells.

When cells were grown at different temperatures and protoplasts from these cells were incubated at 34 °C to regenerate, a somewhat different pattern was observed (Fig. 3).

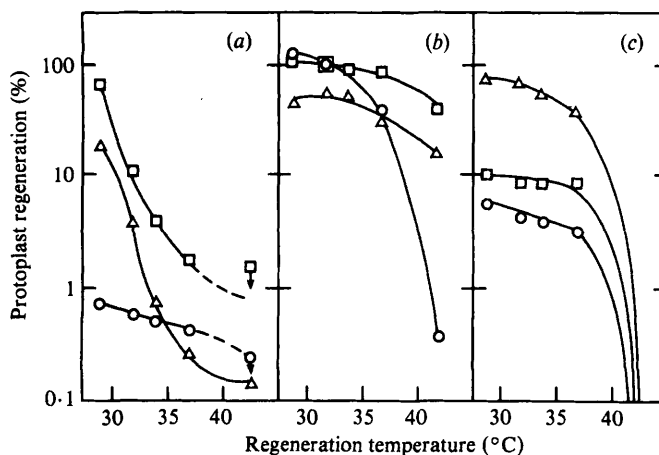


Fig. 4. Effects of temperatures for cell growth and for protoplast regeneration on the efficiency of regeneration with *S. albus* G J1074 (a), *S. ambofaciens* (b) and *S. griseofuscus* (c). Cells were grown in TS broth plus glycine at 29 °C (○), 34 °C (□) and 37 °C (△) before protoplasting, and protoplasts were plated on regeneration media and incubated at various temperatures. The regeneration medium for *S. albus* G was supplemented with isoleucine plus valine at 300 µg ml⁻¹.

Protoplasts prepared from cells grown at 34 °C or below regenerated at about 20% of maximum efficiency, while protoplasts prepared from cells grown at higher temperatures regenerated at progressively lower efficiencies.

When protoplasts were regenerated at the same temperatures as used for cell growth, cell regeneration from protoplasts was relatively efficient at 32 °C or below, but was extremely inefficient (i.e. 0.01%) at 37 °C or above. These observations indicate that both the temperatures for cell growth and for protoplast regeneration are very important, and the negative effects of elevated temperatures appear to be synergistic. In addition, growing cells at low temperature can only partially offset the negative effects of regenerating cells at high temperature, and vice versa. Curiously, *S. fradiae* grows most rapidly at about 37 °C (data not shown).

Effects of temperature on regeneration of protoplasts of other Streptomyces species

Streptomyces albus G J1074, *S. ambofaciens* and *S. griseofuscus* cells were grown at 29 °C, 34 °C and 37 °C in TS broth plus glycine. Protoplasts were prepared, plated on regeneration medium, and incubated at various temperatures. Protoplasts of *S. albus* G J1074 prepared from cells grown at 34 °C regenerated at nearly 100% efficiency when incubated at 29 °C (Fig. 4). Protoplasts from *S. albus* cells grown at 37 °C also regenerated fairly efficiently (about 20%) at 29 °C, but protoplasts from cells grown at 29 °C regenerated very poorly (about 1%) at 29 °C. In all cases, the regeneration efficiencies declined as the temperatures for regeneration increased.

Protoplasts of *S. ambofaciens* prepared from cells grown at 29 °C or 34 °C regenerated at essentially 100% efficiencies when incubated at 29 °C, while protoplasts prepared from cells grown at 37 °C regenerated at about 50% efficiency at 29 °C. Protoplasts from *S. ambofaciens* cells grown at 29 °C regenerated much less efficiently at 42 °C than those from cells grown at 34 °C or 37 °C.

Protoplasts of *S. griseofuscus* prepared from cells grown at 37 °C regenerated viable colonies at nearly 100% efficiency at 29 °C, while protoplasts from cells grown at lower temperatures regenerated less efficiently at 29 °C. The efficiency of regeneration of all three protoplast preparations declined slightly with increasing temperature up to about 37 °C, then declined precipitously at 42 °C.

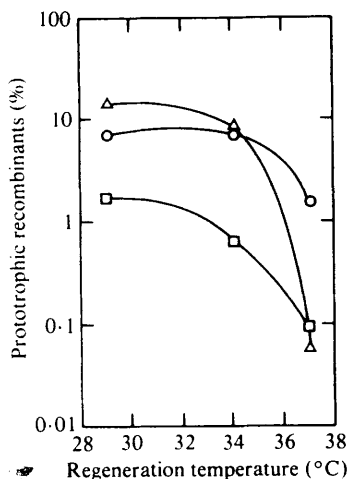


Fig. 5

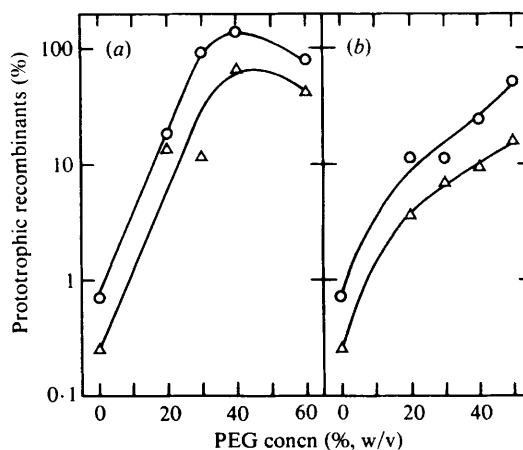


Fig. 6

Fig. 5. Effects of temperatures for cell growth and for protoplast regeneration on recombination frequencies. Cells of *S. fradiae* AR11 (*cysD1*) and AR60 (*metA17*) were grown at 29 °C (O), 34 °C (Δ) and 37 °C (□) before protoplasting, and protoplasts were fused by treatment with 50% PEG 1000. Prototrophic recombinant frequencies were determined by Method 1.

Fig. 6. Effects of PEG concentration and molecular weight on recombination frequencies with *S. fradiae* AR11 and AR60. Recombinant frequencies were determined by Method 1 (O) and Method 2 (Δ). The PEG concentrations given are those before mixing with protoplasts; the concentrations after mixing were 10% lower. (a) PEG 1000; (b) PEG 6000.

Protoplasts from all three species were also capable of regenerating confluent lawns of cells when high concentrations of protoplasts were plated in soft agar overlays on the partially dehydrated regeneration medium.

Regeneration efficiencies have been determined in numerous separate experiments with the four species of *Streptomyces* discussed above, and the efficiencies obtained under optimum conditions (Figs 3 and 4) have been very reproducible. Note, however, that all experiments were carried out with protoplasts prepared from mycelia which were grown to just beyond the late-exponential growth phase (i.e. to an A_{600} of between 2.0 and 7.0). It has been shown previously (Baltz, 1978) that protoplasts prepared from *S. fradiae* or *S. griseofuscus* grown to this phase of growth regenerated most efficiently. We have not explored the effects of temperature on regeneration of protoplasts prepared from mycelia harvested from earlier phases of growth.

Effects of temperature on recombination frequencies after protoplast fusions with S. fradiae

Auxotrophic mutants of *S. fradiae* (AR11 and AR60) were grown at several different temperatures before protoplast formation. Protoplasts from these strains were induced to fuse by PEG treatment; fused protoplasts were plated on unsupplemented and supplemented regeneration medium and then incubated at different temperatures. Prototrophic recombinant frequencies were determined by Method 1. Figure 5 shows that protoplasts from cells grown at 29 °C or 34 °C produced equally high recombinant frequencies when fused protoplasts were incubated at 29 °C or 34 °C; recombinant frequencies were reduced when fused protoplasts were incubated at 37 °C. Also, protoplasts prepared from cells grown at 37 °C yielded reduced recombinant frequencies, even when fused protoplasts were incubated at lower temperatures.

Effects of PEG concentration on recombination frequencies after protoplast fusions with S. fradiae

In the initial protocol developed for *S. fradiae*, protoplast fusion was induced by treatment of protoplasts with 40% PEG 6000 (Baltz, 1978). More recently, Hopwood & Wright (1979) have shown that 50% PEG 1000 gives maximum recombination frequencies (10–20%) in *Streptomyces coelicolor*. They determined recombinant frequencies among the progeny of fused protoplasts which regenerated as confluent lawns on non-selective regeneration medium. (We refer to this as the 'confluent lawn' technique or Method 3.) Ochi *et al.* (1979) have shown that treatment of *Streptomyces parvulus* protoplasts with 65% PEG 4000 gives maximum recombination frequencies of about 40%. They calculated recombinant frequencies by diluting fused protoplasts and comparing the numbers of prototrophic colonies on unsupplemented plates to the numbers of colonies on plates supplemented with the nutritional requirements of the parental strains. (We refer to this as the 'serial dilution' technique.)

We have compared the frequencies of prototrophic recombinants obtained by treating protoplasts of *S. fradiae* auxotrophic strains with PEG 1000 and PEG 6000 at various concentrations. We used 'serial dilution' techniques (i.e. Methods 1 and 2) since *S. fradiae* does not sporulate well, and substrate mycelia must be analysed in the 'confluent lawn' technique (Method 3). The additional steps involved in Method 3 with *S. fradiae* make it intrinsically less accurate. To calculate recombinant frequencies by the 'serial dilution' techniques, we plated fused protoplasts on unsupplemented regeneration medium for prototrophic recombinants and on regeneration medium containing the required nutrients of one, the other, or of both parents. Recombinant frequencies were calculated as the frequency of prototrophic colonies among the total colonies derived from fused protoplasts (Method 1), or as the ratio of prototrophic colonies to the total colonies derived from either parent (Method 2). With the latter method, aggregates containing more than one parent should have been counted twice. Figure 6 shows the results of a typical experiment designed to evaluate the efficiency of protoplast fusion induced by various concentrations of PEG 1000 and PEG 6000. With either method, the frequency of prototrophic recombinants induced by PEG 1000 increased with increasing PEG concentration to a maximum with 40 to 60% PEG. The recombinant frequency calculated by Method 1 was essentially 100%, suggesting that each aggregate of protoplasts yielded prototrophic recombinants. With Method 2, the calculated recombinant frequency was reduced about twofold at high PEG concentrations, suggesting that both parental protoplasts were in fact present in most aggregates (see below).

With PEG 6000 treatment, prototrophic recombinant frequencies calculated by Method 1 increased with increasing concentration to a maximum of about 50% at 50% PEG. Higher PEG concentrations were not tested because of high viscosities. The maximum recombinant frequency was about 15% when calculated by Method 2.

Figure 7 shows the loss of colony-forming protoplast units as a function of PEG concentration in the experiment described in Fig. 6. With PEG 1000, a maximum sixfold reduction in colony formation was obtained at 30% PEG or above, suggesting that the average aggregate consisted of about six protoplasts. Thus, the maximum frequency of prototrophic recombinants per initial (unfused) viable protoplast obtained with PEG 1000 treatment (Fig. 6) was about 15%.

With PEG 6000 treatment, a maximum threefold reduction in colony-forming units was obtained at PEG concentrations of 20% and above, suggesting that the average aggregate contained about three protoplasts. Thus, the maximum frequency of prototrophic recombinants per initial viable protoplast obtained by treatment of protoplasts with 50% PEG 6000 was also about 15%.

Similar experiments have been repeated several times, and in all cases, optimum recombinant frequencies have been achieved with 40 to 60% PEG treatment. In some cases, recombinant frequencies determined by Method 1 have been up to tenfold lower than the results presented in Fig. 6 (see Fig. 5, for instance). In these cases, either the ratio of viable

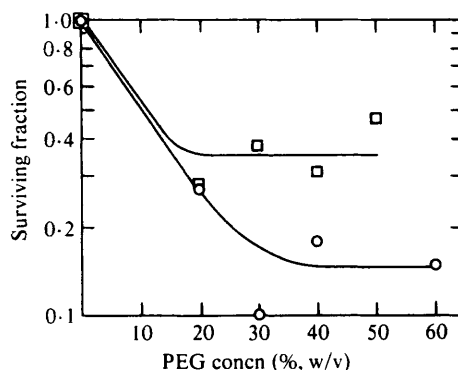


Fig. 7. Effects of PEG treatment on colony formation by *S. fradiae* protoplasts. Protoplasts of *S. fradiae* AR11 and AR60 used to determine recombinant frequencies (Fig. 6) were plated on regeneration medium supplemented with methionine plus cysteine to determine total viable protoplast aggregates after PEG treatment. □, PEG 6000 treatment; ○, PEG 1000 treatment.

parental protoplasts deviated substantially from unity or an unusually high percentage of protoplasts remained unfused as judged by very high survival of protoplasts after PEG treatment. In these cases, however, the frequencies of genetic recombinants per initial viable minority parent remained high (i.e. 10–20%).

In several experiments, recombinant frequencies have been determined by both the 'serial dilution' and 'confluent lawn' techniques. The frequencies of prototrophic recombinants calculated by the latter technique (Method 3) were generally two- to fivefold lower than those calculated by Method 1, as expected, and were usually in the range of 10 to 20% under optimum fusion conditions (data not shown).

DISCUSSION

Identification of methods to form protoplasts and regenerate viable cells from protoplasts of *Streptomyces* has enabled the development of highly efficient techniques for genetic recombination by protoplast fusion and DNA transformation for gene cloning. Protoplasts of certain *Streptomyces* species, however, regenerate asynchronously under standard conditions (Hopwood *et al.*, 1977; Baltz, 1978), and colonies which develop early produce auto-inhibitory substance(s) which inhibit regeneration of surrounding protoplasts. With *S. fradiae*, a species which produces an economically important macrolide antibiotic, tylosin (Seno *et al.*, 1977), auto-inhibition was particularly severe, and only about 1000 colonies could develop on modified R2 agar plates, even when as many as 10^5 protoplasts potentially capable of regeneration were plated. The lack of confluent lawn development has hindered genetic analysis in *S. fradiae* (Baltz, 1980). In addition, severe auto-inhibition may severely constrain gene cloning in species which express it.

In this communication we have shown that auto-inhibition in *S. fradiae* can be essentially eliminated by dehydrating the modified R2 agar plates prior to use and by plating protoplasts in soft agar overlays. Dehydration of regeneration medium by 15 to 22% resulted in more rapid, more synchronous and more efficient regeneration of *S. fradiae*. Concentration of nutrients by equivalent percentages did not alter regeneration, suggesting that dehydration of the surface of the agar medium is most important. One consequence of surface dehydration might be rapid dehydration of soft agar overlays, which might in turn minimize protoplast lysis and provide an environment suitable for rapid cell wall regeneration. Also, dehydration might retard the diffusion of auto-inhibitory substances from developing colonies, particularly if they are autolytic proteins.

The efficiency of regeneration of cells from protoplasts was influenced by the incubation

temperatures for cell growth (prior to protoplast formation), and for cell regeneration from protoplasts. The temperature regimen for *S. fradiae* which yielded essentially 100% regeneration was to grow cells at 29 °C and to regenerate protoplasts at 29 °C. Cells grown at higher temperatures yielded protoplasts which regenerated less efficiently at 29 °C. Also cells grown at 29 °C yielded protoplasts which regenerated very poorly at elevated temperatures. Protoplasts prepared from cells grown at elevated temperatures (34 °C or higher) regenerated even less efficiently at elevated temperatures than those grown at 29 °C, further supporting the conclusion that both the temperatures for cell growth and for protoplast regeneration are critical. Also, these optimum temperatures for efficient regeneration are substantially lower than the temperature (37 °C) which gives the maximum rate of mycelial growth in TS broth.

Protoplasts from three other species of *Streptomyces* also regenerated most efficiently at 29 °C (or below). However, each species had a different optimum temperature for cell growth (prior to protoplast formation) to ensure efficient regeneration. Protoplasts of *S. albus* G J1074 prepared from cells grown at 34 °C regenerated at about 80% efficiency at 29 °C. Protoplasts of *S. ambifaciens* prepared from cells grown at 29 °C or 34 °C regenerated at essentially 100% efficiency, while protoplasts of *S. griseofuscus* prepared from cells grown at 37 °C regenerated more efficiently than those prepared from cells grown at lower temperatures. Clearly, the physiological state of the protoplasts prior to regeneration is important for efficient regeneration. The mechanism involved has not been investigated.

Note also that all experiments described in this report were carried out with protoplasts prepared from mycelia grown to just beyond the exponential growth phase. It was shown previously that protoplasts from mycelia harvested during this growth phase regenerated more efficiently than protoplasts prepared from mycelia in the mid- to late-exponential or late-stationary growth phases (Baltz, 1978).

We have shown also that the incubation temperatures for cell growth and for protoplast regeneration can influence the frequencies of genetic recombinants obtained among regenerated mycelia after protoplast fusion. The highest recombinant frequencies were obtained with *S. fradiae* when cells were grown at 29 °C or 34 °C and protoplasts were regenerated at these temperatures. Protoplasts from cells grown at 37 °C formed recombinants poorly at 29 °C or 34 °C, and even less efficiently at 37 °C. In the latter case, the recombinant frequency was 100-fold lower than the 10% obtained under optimum conditions. Since these protoplasts regenerated at about 0.01% efficiency (see Fig. 3), the overall yield of genetic recombinants per initial parental protoplast was reduced 10⁶-fold by carrying out the growth and regeneration phases at 37 °C rather than at 29 °C. These results demonstrate that protoplasts which are capable of efficient cell regeneration are also capable of efficient fusion, and can produce genetic recombinants at very high frequencies. Conversely, protoplasts which regenerate poorly either do not fuse efficiently or form fusion complexes which are even more unstable than unfused protoplasts, and yield lower frequencies of genetic recombinants.

Our studies have shown that with the modified conditions for highly efficient protoplast fusion and regeneration, highest recombinant frequencies were obtained by treating *S. fradiae* protoplasts with 40 to 60% PEG 1000. This is consistent with previous data obtained with *S. coelicolor* (Hopwood & Wright, 1978, 1979), which showed that maximum recombinant frequencies were obtained with 50% PEG 1000 treatment. It is likely, from data presented in Fig. 6 and from additional data not shown, that 50% PEG 1000 also gives optimum recombinant frequencies in *S. fradiae*.

The protoplast fusion and transformation techniques developed in *Streptomyces* should be broadly applicable for genetic analysis and manipulation of strains of this economically important genus (Queener & Baltz, 1979). The results reported here have identified several factors which influence the efficiencies of protoplast regeneration and fusion, and should be useful in applications of these methodologies to other *Streptomyces* species.

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