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CHAPTER 4

Genetic Recombination by Protoplast Fusion in *Streptomyces*

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Genetic recombination by protoplast fusion recently has been demonstrated in a number of *Streptomyces* species, and in at least one species of *Streptosporangium*. Protoplast formation by lysozyme treatment is facilitated by growing mycelia in a complex medium containing a partially growth inhibitory level of glycine. Protoplast fusion is readily induced by treating protoplasts with polyethylene glycol, and regeneration of cells from protoplasts can take place on hypertonic media. The efficiency of cell regeneration from protoplasts of *Streptomyces fradiae* and *Streptomyces griseofuscus* is growth-phase dependent; maximum regeneration is obtained from cells harvested between late exponential and stationary growth phases. Recombinant frequencies in intraspecific streptomycete crosses by protoplast fusion are very high and typically vary from about 0.5 to 15% of total viable protoplasts. The frequency of recombinants can be further increased in *Streptomyces coelicolor* and *S. fradiae* (and presumably in other *Streptomyces*) by treating protoplasts with ultraviolet (UV) light. The very high frequencies of recombinants obtained should facilitate construction of superior antibiotic-producing strains without genetically marking the parental strains. It also should facilitate construction of strains with complex genotypes for intraspecific genetic mapping studies and may facilitate formation of interspecific streptomycete hybrids to produce novel antibiotics. Protoplast fusion currently is being used to construct a genetic map of *S. fradiae*, and preliminary experiments indicate that the *tylA* gene, which codes for an enzyme involved in formation of a common intermediate for biosynthesis of the tylosin sugars, is chromosomal.

INTRODUCTION

The prokaryotic genus *Streptomyces* is curious if not phenomenal in its ability to synthesize antibiotics of diverse chemical structure and mode of action (Hopwood and Merrick 1977). Of the 3,000 or so antibiotics discovered through about 1974, about 70% are produced by *Streptomyces* (Hopwood and Merrick 1977).

Genetic manipulation of these economically important microorganisms has commercial applications in yield improvement and in discovery of new intermediates and hybrid antibiotics with beneficial properties (Queener and Baltz 1979). Both of these applications can be best achieved if one has available a number of genetic techniques (Queener and Baltz 1979). However, two essential techniques are efficient mutation and general recombination.

Prior to 1977, conjugation was the only successful technique to effect genetic recombination in *Streptomyces* (Hopwood and Merrick 1977). While conjugation is fairly common among *Streptomyces* (Hopwood and Merrick 1977), recombinant frequencies are often very low or undetectable in some strains. Since numerous different species of *Streptomyces* produce antibiotics of economic interest, it seemed apparent that a more general technique to yield high frequencies of recombinants in a variety of *Streptomyces* would be very useful.

Since 1977, three new recombination techniques have been developed for *Streptomyces:* transduction (Stuttard 1979); protoplast fusion (Hopwood et al. 1977; Baltz 1978); and protoplast transformation (Bibb et al. 1978). All of these techniques have specific advantages and applications for particular genetic manipulations in *Streptomyces* (Queener and Baltz 1979). The methodology, advantages, and applications of the new general recombination technique of protoplast fusion are discussed in this paper.

Materials and Methods

General methods for protoplast fusion. The media, growth conditions, procedure for ultrasonic fragmentation of mycelia, and conditions for formation, regeneration, and fusion of protoplasts have been described (Baltz 1978).

Other media. AS-1 medium contained per liter deionized H_2O: yeast extract, 1 g; L-alanine, 0.2 g; L-arginine, 0.2 g; L-asparagine, 0.5; soluble starch, 5 g; NaC1, 2.5 g; Na_2SO_4, 10 g; agar, 20 g. The pH was adjusted to 7.5.

CDA medium contained per liter deionized H_2O: Czapek Dox broth (Difco), 35 g; L-asparagine, 2 g; Difco agar, 15 g.

Strains. Derivatives of *S. fradiae* C373, which produces the macrolide antibiotic tylosin, and *S. griseofuscus* were used in this study and have been described previously (Baltz 1978). Mutants blocked in tylosin biosynthesis and containing various auxotrophic mutations were isolated after N-methyl-N′-nitro-N-nitrosoguanidine mutagenesis (Seno, Radue, and Baltz, unpubl. data) by a procedure similar to that of Delić et al. (1970).

Treatment of protoplasts with ultraviolet light. S. fradiae M1 (Baltz 1978) was grown in TS broth plus 0.4% glycine to an A_{600} of 3.4. A portion of the mycelia was diluted fourfold and treated with ultrasound (Baltz 1978) at 76 watts for 13 sec to obtain mycelial fragments containing predominately single cells. The cells were washed twice in medium P and resuspended cells were homogenized (Baltz 1978) to eliminate clumping. A second portion of mycelia was used to prepare protoplasts as described by Baltz (1978).

Portions of washed mycelial fragments (cells) and washed protoplasts in medium P were either (a) diluted 10-fold in medium P, or (b) treated with PEG as described (Baltz 1978), then diluted 10-fold in medium P. The preparations of PEG-treated and untreated cells or protoplasts were gently swirled in Petri dishes with magnetic agitation, and treated with ultraviolet (UV) light at 240 $\mu W/cm^2/sec$. Samples were withdrawn at various times, diluted, and plated on R2 medium for viable counts. Manipulations were carried out in semi-dark to avoid photoreactivation.

Linkage and gene sequence analysis. Protoplasts of multiply marked strains of *S. fradiae* were fused by PEG treatment and plated on modified R2 medium containing all but one of the nutritional requirements of each parent. Recombinants prototrophic for the other two loci were allowed to regenerate, and the developing colonies were picked and streaked on the same medium. Isolated colonies which developed after several days at 34 C were picked and patched on CDA plates containing all combinations

of appropriate supplements to score nonselected allele frequencies. Linkage and gene sequence analyses were carried out as described (Hopwood 1972).

Results and Discussion

Streptomyces *Protoplasts*

Protoplast formation. The protoplast fusion technique requires formation of stable protoplasts, fusion of protoplasts to allow recombination, and regeneration of viable cells from fused protoplasts.

Sagara et al. (1971) reported that *Streptomyces griseoflavus* grown on a medium containing a high concn of glycine was sensitive to lysozyme treatment and formed protoplasts. Okanishi et al. (1974) demonstrated that *Streptomyces griseus* and *Streptomyces venezualae* grown in a medium containing a partially inhibitory concn of glycine became susceptible to rapid and efficient protoplast formation by treating with lysozyme and lytic enzyme No. 2 in hypertonic medium. More recently, this general technique has been applied to a variety of other *Streptomyces* (Hopwood et al. 1977; Baltz 1978).

Figure 1 shows the growth kinetics of *S. fradiae* and *S. griseofuscus* in trypticase soy broth with and without glycine. With these species, roughly twofold reductions in

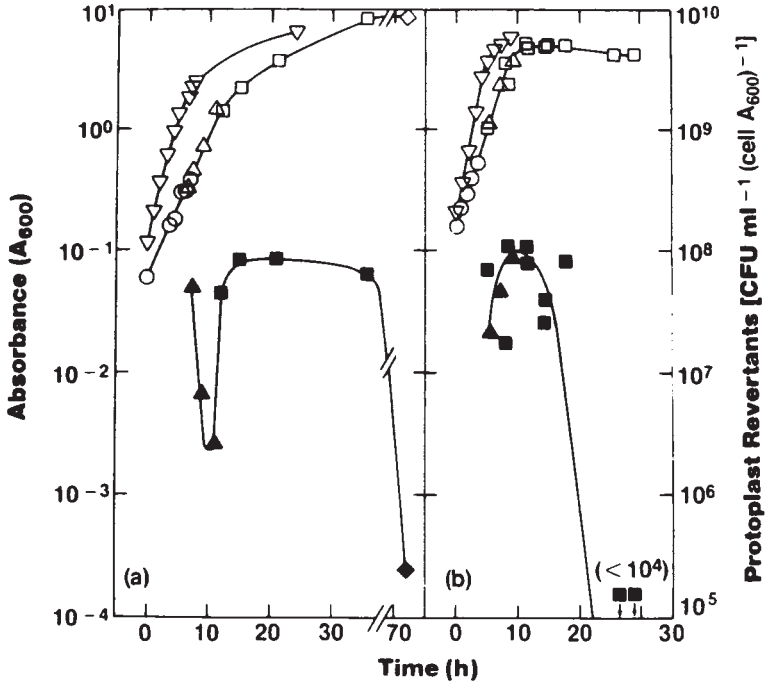

FIG. 1. Growth of *S. fradiae* and *S. griseofuscus* and regeneration of protoplasts as a function of growth phase. (a) *Streptomyces fradiae* MI was grown in TS broth (▽) or TS broth plus 0.4% glycine (○, △, □, ◇). The different symbols represent data from different experiments which have been superimposed to define the complete growth cycle. Mycelial fragments from one experiment (□) were refrigerated overnight before protoplast formation. Closed symbols represent the number of protoplast revertants per unit A_{600} of cells taken from the corresponding growth phase (with glycine). (b) *Streptomyces griseofuscus* was grown in TS broth (▽) or TS broth plus 0.8% glycine (○, △, □). Again, data from different experiments have been superimposed. Closed symbols represent the number of protoplast revertants per A_{600} of cells taken from the corresponding growth phase (with glycine).

growth rate were achieved by adding 0.4 and 0.8% glycine, respectively. With *Streptomyces cinnamonensis*, about 2% glycine was required to achieve a similar reduction in growth rate (not shown). Addition of enough glycine to diminish the growth rate by about twofold has rendered these and other species of *Streptomyces* very susceptible to protoplast formation by lysozyme treatment alone. The protoplasts formed are stable in medium P (Okanishi et al. 1974), but may loose viability upon storage at 4 C (see below). Hopwood and Wright (1979) also have found recently that lysozyme treatment alone is sufficient to produce recombination-proficient protoplasts from *S. coelicolor* grown in a medium containing glycine.

Regeneration of cells from protoplasts. Okanishi et al. (1974) demonstrated that protoplasts of *S. griseus* and *S. venezualae* were capable of regenerating viable cells on hypertonic medium (R2 medium) containing calcium and magnesium ions to stabilize the protoplasts. More recently, it has been shown that protoplasts from many other *Streptomyces* species can regenerate viable cells on R2 medium (Hopwood et al. 1977) or on modified R2 medium containing asparagine instead of proline (Baltz 1978). The efficiency of cell regeneration from protoplasts, however, is dependent upon the growth phase in glycine-containing medium (Baltz 1978). Figure 1 shows that with *S. fradiae* and *S. griseofuscus* cell regeneration is most efficient with protoplasts prepared from mycelia harvested between exponential and stationary growth phases. Nearly 10^8 colony-forming protoplasts/ml were liberated from each A_{600} unit of mycelia. This represents efficiencies of roughly 50 and 100% per protoplast for *S. fradiae* and *S. griseofuscus*, respectively (Baltz 1978). The efficiency of regeneration declines dramatically after cells enter stationary phase. With *S. fradiae*, cells from early exponential growth phase also regenerate very efficiently, but the efficiency declines as cells enter mid-to late exponential growth phase.

Regeneration of *S. griseofuscus* is very rapid, and visible colonies develop within 2 days. In addition, if large numbers of protoplasts are spread on modified R2 agar, confluent lawns of cells develop. With *S. fradiae* regeneration is slow and asynchronous, and the colonies which develop early inhibit regeneration of nearby protoplasts (Baltz 1978). Hopwood et al. (1977) have observed a similar auto-inhibitory phenomenon with *Streptomyces acrimycini*, while *S. coelicolor, Streptomyces parvulus, Streptomyces lividans*, and *S. griseus* will regenerate confluent [or nearly confluent (Hopwood and Wright 1979)] lawns of cells.

The high efficiency of regeneration obtained indicates that protoplasts prepared in this manner are quite stable and can withstand the multiple centrifugations and resuspensions required during the washing procedure (Baltz 1978). Protoplasts of *S. fradiae* and *S. griseofuscus* have been stored at 4 C for various lengths of time to determine the feasibility of using the same stock of protoplasts for multiple experiments over a 1 to 2-wk period. In general, *S. griseofuscus* protoplasts lose viability much more rapidly than *S. fradiae*, and the rate of loss appears to depend somewhat on the physiological state of the mycelia before protoplast formation. Two- to 10-fold losses in viability in 2 days and 10^3- to 10^4-fold losses by 12 days are common. *S. fradiae* protoplasts show somewhat bizarre oscillations in viability as a function of storage time, and in some cases *gain* viability during storage before ultimately losing viability. Up to 10-fold increases in viability were seen when poorly regenerating protoplasts from mid- to late exponential growth phase were stored at 4 C for about 7 days. By 12 to

13 days, however, the viability had declined by 10- to 100-fold. It is tempting to speculate that the *S. fradiae* protoplasts may continue to metabolize at 4 C to a certain extent, and that protoplasts may proceed through different physiological phases with respect to regeneration efficiency. The combined results with these two species, however, suggest that it may be prudent to use protoplasts freshly prepared from mycelia harvested during the transition from exponential to stationary growth phases to assure consistent results.

Recombination by Protoplast Fusion

General methodology. Two similar techniques to effect genetic recombination by protoplast fusion in *Streptomyces* have been developed independently. The method described by Hopwood et al. (1977) was modeled after a technique used to fuse mammalian cells (Pontecorvo et al. 1977), while that described by Baltz (1978) is similar to that used in *Bacillus* protoplast fusions (Fodor and Alföldi 1976; Schaeffer et al. 1976). The technique described by Baltz (1978) employed 40% polyethylene glycol (PEG) as a fusing agent, while that of Hopwood et al. (1977) employed 40% PEG plus 15% dimethylsulfoxide (DMSO) or 50% PEG. A more detailed analysis of optimum fusion conditions has recently shown that DMSO is not required for efficient fusion and recombination (Hopwood and Wright 1979). In *S. coelicolor* recombinants are generally recovered after regeneration of fused protoplasts in confluent lawns on R2 medium (Hopwood et al. 1977; Hopwood and Wright 1978, 1979), while in *S. fradiae* recombinants are recovered as individual colonies (Baltz 1978) since auto-inhibition precludes development of confluent lawns. The general technique of protoplast fusion has been extended to a number of other *Streptomyces* which are listed in Table 1. It is now apparent that protoplast fusion is, in fact, broadly applicable to induce very high frequencies of genetic recombinants in many *Streptomyces*, including strains cured of known fertility plasmids and other strains in which conjugal recombination is marginal or completely lacking. Furthermore, extension of this methodology to *Streptosporangium* suggests that protoplast fusion may soon be applied to genetically manipulate other *Actinomycetales* such as *Nocardia* and *Micromonospora* which produce interesting antibiotics (Hopwood and Merrick 1977).

Enhancement of recombination frequencies by UV light. Hopwood and Wright (1979) have shown that even higher frequencies of genetic recombinants can be obtained if parental protoplasts are treated with UV light. Two- to 10-fold increases in recombinant frequencies were obtained when protoplasts were inactivated to about 1% survival by UV light. We have obtained similar results with *S. fradiae*. Figure 2 shows the UV inactivation dose-response of *S. fradiae* cells and protoplasts with and without PEG treatment. The inactivation slopes for PEG-treated and untreated cells were quite similar. PEG treatment of cells, however, reduced the zero time viable counts, presumably by causing cell clumping. Untreated protoplasts appeared to be a little more sensitive to UV than PEG-treated or untreated cells. PEG-treated protoplasts, however, showed a marked reduction in inactivation slope, suggesting that the fusion process allows reactivation of UV-inactivated protoplasts. Similar results were obtained when protoplasts were treated with UV prior to PEG-treatment. Also, when strains AR30 (*metA5, arg-2, spc-2*) and AR11 (*cysD1*) (Baltz 1978) were crossed by protoplast fusion, prior

TABLE 1. *Recombinant frequencies obtained by conjugation and protoplast fusion*

Strains[a]	Recombinant Frequencies: Conjugation	Recombinant Frequencies: Protoplast Fusion	Reference
Streptomyces acrimycini	3.2×10^{-5}	[b] 2.6×10^{-4}	Hopwood et al. (1977)
Streptomyces antibioticus	—	[c] 1.5×10^{-1}	Katz (pers. comm.)
Streptomyces coelicolor (SCPI$^-$ SCP2$^+$)	1.7×10^{-6}	4.7×10^{-2}	Hopwood et al. (1977)
Streptomyces coelicolor (SCPI$^-$ SCP2$^-$)	1.7×10^{-7}	6.2×10^{-2}	Hopwood et al. (1977)
Streptomyces fradiae	$<1.0 \times 10^{-7}$	[d] $>6.0 \times 10^{-3}$	Baltz (1978)
Streptomyces fradiae	—	5.6×10^{-2}	Godfrey et al. (1978)
Streptomyces griseofuscus	—	$>4.2 \times 10^{-4}$	Baltz (unpubl. data)
Streptomyces griseus	2.3×10^{-6}	1.0×10^{-2}	Hopwood et al. (1977)
Streptomyces lactamdurans	10^{-6}–10^{-2}	1.7×10^{-1}	Wesseling et al. (1978)
Streptomyces lividans	$<1.0 \times 10^{-6}$	6.0×10^{-2}	Hopwood et al. (1977)
Streptomyces parvulus	6.3×10^{-6}	6.0×10^{-3}	Hopwood et al. (1977)
Streptomyces parvulus	—	1.5×10^{-1}	Katz (pers. comm.)
Streptosporangium viridogriseum	5.0×10^{-5}	1.0×10^{-2}	Oh, Speth, and Nash (this volume)
Streptomyces fradiae × *Streptomyces bikiniensis*	$<1.0 \times 10^{-9}$	1.7×10^{-5}	Godfrey et al. (1978)

[a] All crosses are intraspecific unless indicated.

[b] This frequency is apt to be an underestimate because of auto-inhibition during regeneration (see Hopwood et al. 1977).

[c] These include many unstable heterokaryons or heteroclones.

[d] This frequency is the most conservative estimate and was calculated from a cross between *cysD* and *metA* loci which are now known to be linked (see Fig. 4 and Baltz 1978).

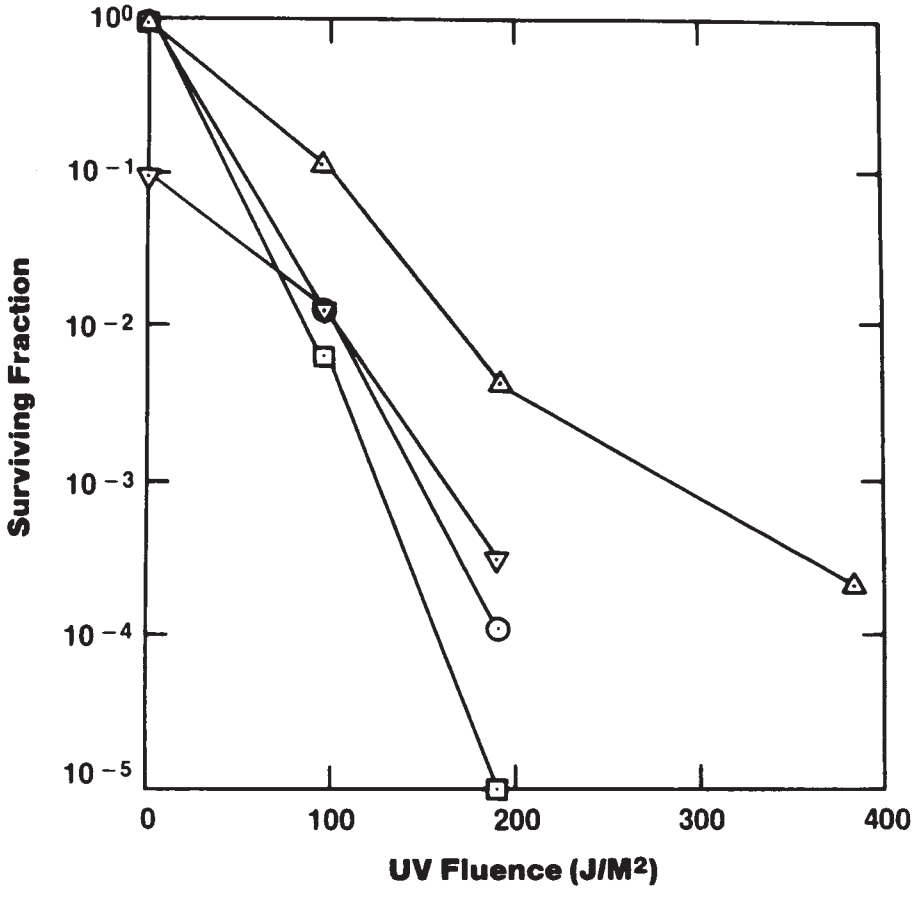

FIG. 2. Reactivation of UV-inactivated protoplasts by PEG-induced fusion. Mycelia of *S. fradiae* Ml were grown to an A_{600} of 3.4. Protoplasts were prepared from one portion of the culture, and the other portion was sonicated for 13 sec to yield mycelial fragments consisting primarily of single cells (Baltz 1978). Surviving fractions of cells and protoplasts are based upon viable counts of untreated cells and protoplasts of 4.4×10^7 and 2.3×10^7/mycelia A_{600} unit, respectively. ○, cells; ▽, PEG-treated cells; □, protoplasts; △, PEG-treated protoplasts. See Materials and Methods for details.

treatment of protoplasts with UV light to between 0.1 and 1.0% survival enhanced the frequency of prototrophic recombinants by about 10-fold (not shown).

In *S. coelicolor* (Hopwood and Wright 1979) the UV treatment appeared to selectively inactivate single (unfused) protoplasts and also to stimulate crossing over. Hence fused protoplasts, which are transiently diploid on the average (Hopwood and Wright 1978), appear to have a survival advantage after UV treatment since they can recombine out potentially lethal damage which may not be repairable in haploid protoplasts; thus, survivors of UV treatment should necessarily be enriched for recombinants. The significance of these observations and the general high frequency of recombination obtained even without UV treatment will be discussed below.

Heteroclone formation. We have recently observed very high frequencies (i.e., >90%) of unstable Met^+ colonies arising on modified R2 medium (lacking methionine but

supplemented with the other amino acids required for growth of the parental strains) after fusion of strains carrying *metA* and *metC* mutations (see Fig. 4 for map locations). Upon serial recloning on the same medium, large Met$^+$ colonies continued to segregate clones of variable colony size including a minority of large and a majority of petite colonies. These complementing mutations appear to be closely linked, and thus the unstable colonies are likely to be heteroclones, since selection for complementation of closely linked markers minimizes recombination and enriches for heteroclones in *Streptomyces* (Hopwood 1967) and tandem duplications in enteric bacteria (Anderson and Roth 1977). The unstable Met$^+$ colonies currently are being analysed to determine if they are indeed heteroclones. Applications of protoplast fusion-induced heteroclones are discussed below.

Applications of Protoplast Fusion

Intraspecies recombination. Formation of intraspecies recombinants can be applied to construct high yielding production strains by crossing strains derived in separate lineages (Hopwood 1977, 1979) and to construct complex genotypes to facilitate genetic mapping (see below). Both of the applications should be facilitated by protoplast fusion for at least three reasons. First, protoplast fusion now appears to be generally applicable in *Streptomyces*, and thus should allow recombinant formation within industrially important species of *Streptomyces* which were not previously amenable to genetic manipulations other than by mutation. Second, the primary product of fusion is, on the average, a transient diploid protoplast (Hopwood and Wright 1978), and thus all markers should participate more or less equally in recombinational events. Furthermore, this transient diploidy allows very long stretches of chromosomal sequences from both parents to be incorporated in recombinants (Hopwood and Wright 1978; Baltz, unpubl. data). This differs dramatically from sex factor-mediated conjugation, which allows expression of certain markers much more frequently than others, and from transduction and transformation which generally allow only a small fraction of the donor chromosome to participate in a given recombinational event. Third, high frequencies of recombinants are generally obtained after fusion, and even higher frequencies can be obtained if protoplasts are treated with UV light. This property of protoplast fusion is particularly advantageous in industrial strain development since it facilitates rapid random screening of recombinants without selection. This eliminates the time-consuming aspects of inducing selectable or counterselectable gene mutations in strains of interest, and, perhaps more importantly, eliminates the problem of accumulation of mutagen-induced cryptic second site mutations which have pleiotropic negative effects on antibiotic productivity. The second site mutations often go undetected since the primary (auxotrophic or drug resistance, etc.) mutations in themselves often exert negative pleiotropic effects on antibiotic production. In addition, Hopwood and Wright (1978) have shown that recombinants containing DNA segments from three or four parents can occur at surprisingly high frequencies after protoplast fusion, so that it is, in fact, feasible to cross more than two parents of different lineages simultaneously without marking any of the strains.

Interspecies recombination. A likely general application of protoplast fusion will be construction of *Streptomyces* hybrid species to produce novel (hybrid) antibiotics

(Queener and Baltz 1979). Fleck (1979) has demonstrated that this is possible through natural conjugation, and Godfrey et al. (1978) (see Table 1) have demonstrated that protoplast fusion can be used to enhance dramatically the frequency of interspecific recombinants in crosses between *S. fradiae* and *Streptomyces bikiniensis*. However, the frequencies of recombinants obtained by protoplast fusion in interspecific crosses were much lower than those obtained in intraspecific crosses (Table 1), as one might expect with strains lacking complete DNA sequence homology, suggesting that random crossing of unmarked strains may be inadvisable. Prior knowledge of antibiotic structural similarities, cosynthetic and bioconversion patterns of intermediates, and map locations of genes involved should be very useful in successful application of this approach.

Genetic mapping. Knowledge of the genetic map locations of antibiotic genes should be very useful for interspecific hybrid formation, for directed mutagenesis in antibiotic structural and regulatory genes, and for gene amplification by classical and recombinant DNA techniques (Queener and Baltz 1979). An obvious question that presents itself with the development of efficient recombination by protoplast fusion is whether this technique can, in fact, be used to construct a genetic map of a previously unmapped streptomycete. We recently have isolated a collection of *S. fradiae* mutants blocked in tylosin biosynthesis and which comprise nine cosynthesis groups (Seno and Baltz 1978; Seno and Baltz, unpubl. data). We have induced additional selectable or counterselectable genetic markers in several of these strains and are currently attempting to map the tylosin genes by protoplast fusion. Our general procedure has been to fuse multiply marked strains and to select for recombination between two specific auxotrophic markers to prototrophy on modified R2 medium supplemented with additional nutritional requirements of parental strains. Recombinant colonies are then picked, recloned on the same medium, then further analyzed for presence of unselected markers. The data are then analyzed to determine linkage relationships and marker sequence (Hopwood 1972). Results from a typical cross are shown in Table 2, and the most likely linkage relationship based upon this and other crosses is shown in Fig. 3. Note that all eight recombinant classes were detected, and the frequency of quadruple crossovers was low (2 of 62). Similar low frequencies of quadruple crossovers have been obtained in other crosses. When these recombinants were further analyzed for the tylosin markers, only 30% of the recombinants produced tylosin or tylosin aglycone, the compound which is produced by *tylA* mutants. Similar high frequency loss (ranging from about 20 to 70%) of tylosin production has been observed in other crosses in which recombinants have been analyzed for expression of *tyl* mutations, and therefore appears to be a general phenomenon associated with some aspect of the protoplast fusion process. The basis for this apparent genetic instability currently is being investigated.

In spite of the high frequency loss of the antibiotic production trait, enough data have been analyzed to date to suggest strongly that the *tylA* gene is chromosomal. Figure 4 shows a preliminary genetic map of *S. fradiae* which summarizes results from many different crosses. The details of the genetic analysis will be published elsewhere.

Our results indicate that protoplast fusion can be used for genetic mapping. Future experiments will focus on mapping of the other tylosin genes to determine if they are chromosomal or extrachromosomal, and if any are clustered in regulatory units. This information will be used to apply directed mutagenesis and amplification of specific

TABLE 2. *Recombinant frequencies obtained after fusion of strains AR26 (metA4, pheAl, spo-1) and AR67(his-4, lys-2, tylA14)*[a]

Recombinant Class	Genotypes					Frequency	Crossovers
	met	lys	phe	his	spo		
1	+	+	+	+	+	5	2
2	+	+	+	+	−	14	2
3	+	+	+	−	+	4	2
4	+	+	+	−	−	1	4
5	+	+	−	+	+	5	2
6	+	+	−	+	−	27	2
7	+	+	−	−	+	5	2
8	+	+	−	−	−	1	4

[a] Recombinants were selected for *met*$^+$,*lys*$^+$ on modified R2 medium containing histidine and phenylalanine.

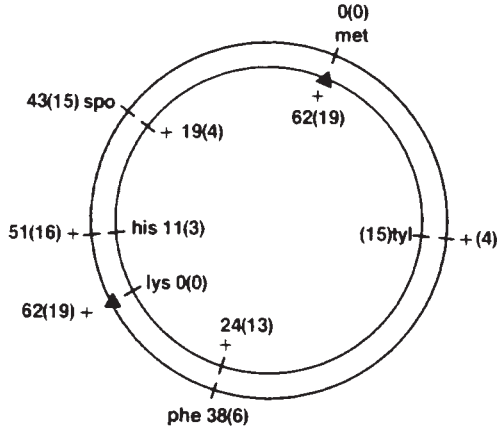

FIG. 3. Sequence of markers involved in protoplast fusion cross between AR26 and AR67. Arrows designate the selected alleles. The numbers indicate frequencies among the recombinants of wild-type and mutant alleles at each locus. Numbers in parentheses indicate the frequency of the various alleles within the subpopulation of recombinants which produce tylosin or tylosin aglycone, the intermediate produced by *tylA* mutants.

genes involved in rate limitation in tylosin production. A key example is the *tylF* gene which codes for macrocin O-methyltransferase. This enzyme carries out the terminal rate-limiting methylation to tylosin (Seno et al. 1977; Seno and Baltz 1978) and has been shown recently to be subject to both substrate and product inhibition (Seno and Baltz, unpubl. data).

Heteroclones. The evidence for heteroclone formation during protoplast fusion is preliminary, but very likely in crosses between closely linked complementing markers in view of previous data from conjugal crosses (Hopwood 1967; Anderson and Roth 1977). Heteroclone analysis (Hopwood 1967) might be used to facilitate genetic mapping and complementation analysis by protoplast fusion. In addition, if heteroclones contain chromosomes with tandem duplications (Anderson and Roth 1977), then they might be exploited to amplify specific regions of the chromosome. Stabilization of such strains,

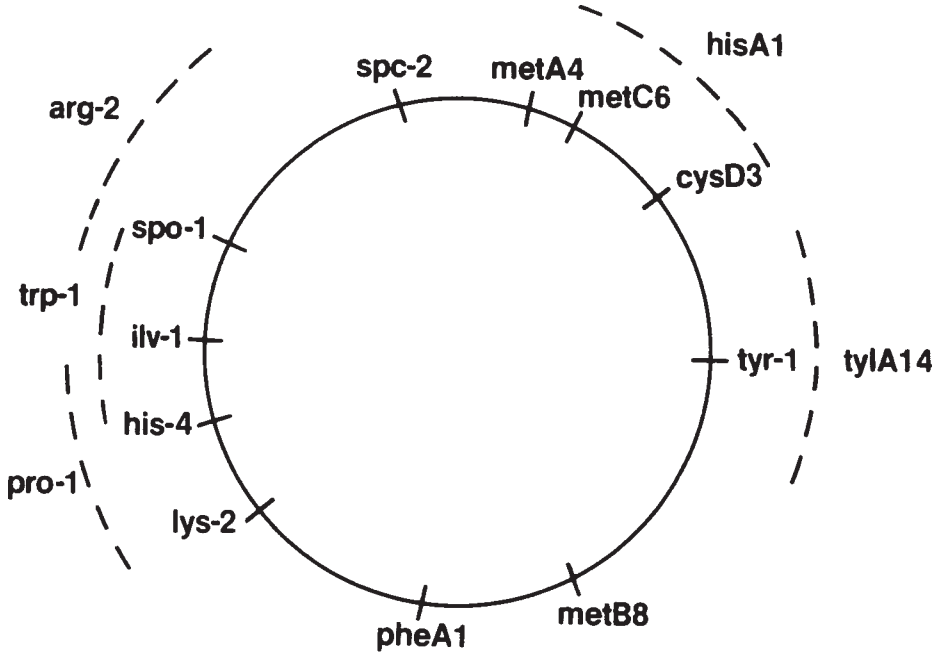

FIG. 4. Preliminary genetic map of *Streptomyces fradiae*. Map locations of markers outside of the main circular map have not yet been ordered with respect to the markers included within the dashed lines. Map distances are only very crude estimates.

however, will likely require the presence of a *recA*-like mutation (Anderson and Roth 1977) which has not yet been identified in *Streptomyces*. Also, it seems likely that heteroclones may comprise a large fraction of primary clones isolated in interspecific crosses between strains of limited base sequence homology (Anderson and Roth 1977). Stabilization of these strains may lead to fruitful applications in hybrid antibiotic production.

Acknowledgment

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