Genetic Interactions in *Streptomyces rimosus* Mediated by Conjugation and by Protoplast Fusion

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(Received 29 September 1982)

The development of a protoplast fusion technique for oxytetracycline-producing *Streptomyces rimosus* strains, and its evaluation for the application for a breeding programme, has been described. Treatment of *S. rimosus* protoplasts with 40% (w/v) PEG 1550 for 30 min gave optimal numbers of recombinants ranging from 1 to 10% of the total progeny. Therefore, by comparison with conjugation, protoplast fusion increased the frequency of recombination by two to three orders of magnitude. The proportion of multiple crossover classes amongst recombinants was higher, by a factor of ten, after protoplast fusion (13.3%) than after conjugation (1.5%). Participation of less frequent complementary genotype doubled from 9.0% in conjugation to 17.9% in protoplast fusion. Overall, this suggested that the opportunities for crossing over in a fusion of *S. rimosus* protoplasts were spatially and/or temporally extended leading to a loosening of linkage with a near-random assortment of genotypes in a cross. However, by minimizing the multiple crossover classes and calculating allele frequency gradients, it was shown that the protoplast fusion technique allows arrangement of genetic markers on the *S. rimosus* chromosome. These are ideal characteristics for the recombination of divergent lines in a strain improvement programme.

INTRODUCTION

Oxytetracycline is produced by wild *Streptomyces rimosus* strains at a very low level (Alačević *et al.*, 1973) and for commercial manufacture it was necessary to increase the product yield. The intensive programmes applied so far involved the isolation of improved genotypes by random mutation and screening, coupled with attempts to optimize fermentation conditions (M. Bošnjak, unpublished results). Since the aim in yield improvement is the accumulation, in one individual, of as many yield-enhancing alleles as possible, recombination between divergent selection lines in a breeding programme should provide a broader gene pool from which to effect selection (Hopwood & Chater, 1980). Although *S. rimosus* has a natural system of genetic recombination through conjugation (Alačević, 1969; Friend & Hopwood, 1971; Alačević *et al.*, 1973), it has been applied to strain improvement to a very limited extent. There are several constraints to effective use of conjugation, not the least being the need to introduce selectable or counterselectable markers into the strains to be crossed.

Techniques exist now for the fusion of protoplasts from a range of *Streptomyces* species by treatment with polyethylene glycol (Hopwood *et al.*, 1977; Baltz, 1978; Godfrey *et al.*, 1978; Ochi *et al.*, 1979). Following protoplast fusion, both the cytoplasm and whole chromosomes of the two cells are brought together in one 'zygote'. Protoplast fusion yields a high proportion of recombinants involving all regions of the genome, with many multiple crossover classes. In addition, it gives a significant proportion of recombinants involving three or four parental genomes (Hopwood & Wright, 1978). Recombination frequencies can be increased even further

by UV irradiation of the parental protoplasts immediately before fusion (Hopwood & Wright, 1979). These are ideal characteristics for the hybridization of divergent lines in a yield improvement programme.

METHODS

Micro-organisms and media. Mutants of *Streptomyces rimosus* ATCC 10970 (R7) 252 (*pro-1 his-4 tyr-1*) and 1054 (*nic-1 rib-4 pdx-9*) were used throughout the study. Propagation and maintenance of stocks, conventional crosses and protoplast fusion were carried out using the media described by Alačević *et al.* (1973) and Pigac *et al.* (1982).

Protoplast preparation and regeneration. Protoplasts were prepared and regenerated according to the 'general' procedure employed by Pigac *et al.* (1982).

Crossing procedure. The conventional procedure for recombination by conjugation used in this study was carried out by the techniques of Alačević *et al.* (1973). Mixed cultures were made on plates of complete medium (CM) overlaid with cellophane discs. After incubation for 5 d at 28 °C, mycelial fragments were scraped off and homogenized. For viability counts, the suspensions were diluted and plated on appropriately supplemented non-selective minimal media (MM), containing growth factors for one or both parents. Recombinant progeny were plated on MM with proline, tyrosine, pyridoxine and nicotinamide, selecting for mutations on two opposite sides of the circular linkage map (*his⁺ rib⁺*). Such selection permitted growth of 25% (i.e. 16 out of 62) possible recombinant genotypes. Although this is the maximum possible number of recombinant classes obtained from a six-factor cross by a single selective medium, it proved satisfactory for comparative purposes. Recombinants were scored by replica-plating of separate colonies on to diagnostic media, or were transferred to master plates and then tested. The recombination frequency was determined as the ratio of recombinant colonies arising on selective plates to the number of colonies arising on supplemented non-selective medium, calculated on the basis of the minority parent.

Protoplast fusion. Purified protoplast suspensions ($1-3 \times 10^9$ c.f.u. ml⁻¹) of two mutants were mixed in 1:1 proportions and pelleted by centrifugation at about 1000 g for 15 min. The protoplast pellet was resuspended in 0.1 ml P medium and mixed with 0.9 ml P medium containing polyethylene glycol (PEG) polymers of 1550, 4000 or 6000 (Serva), previously sterilized by autoclaving, to give concentrations of 20, 40 or 60% (w/v). After 0, 1, 10 and 30 min at 28 °C the PEG-treated protoplasts were diluted with 4 ml P medium, centrifuged at 1000 g for 15 min and resuspended in 1 ml P medium. Appropriate dilutions were plated on selective and non-selective minimal regeneration media (MRM) containing required growth factors and analysed as described. Control preparations, without PEG, were made by suspending the mixed protoplast pellets in 5 ml P medium, centrifuging and resuspending in 1 ml P medium. Appropriate dilutions were plated as indicated above.

RESULTS AND DISCUSSION

Conditions for optimal fusion of protoplasts

The most important factors affecting the frequency of recombination by protoplast fusion are: molecular weight of PEG, its concentration, and the time of treatment used to induce fusion. In order to study the influence of factors affecting fusion frequencies, the 'serial dilution' technique (Baltz, 1978; Ochi *et al.*, 1979) was adopted. Recombination frequencies obtained by protoplast fusion were determined by diluting and plating fused protoplasts on selective media, which allowed growth of the maximum possible number of recombinants from the multiple-factor cross. Direct plating of fused protoplasts on selective medium underestimates the total fusion frequency for a number of reasons (Hopwood, 1981). However, since *Streptomyces rimosus* R7 mutants do not sporulate well, substrate mycelia would have had to be used in the 'confluent lawn' technique (Hopwood *et al.*, 1977; Hopwood & Wright, 1979), which would have made the analysis less accurate. Furthermore, the auxotrophic mutants used were of the initial fertility type and could have confused the results of protoplast fusion by recombination between mycelia after wall regeneration. For this and other reasons, the 'serial dilution' technique proved to be satisfactory (at least for comparative purposes) for assessing fusion frequency in this system.

The optimal conditions for fusion of protoplasts of *S. rimosus* mutants are summarized in Table 1. When PEG was added to the mixture of protoplasts, visible aggregation took place. Aggregation reduced the number of parental regenerants by about 90%. Therefore, the frequency of recombination was calculated on the basis of the minority parent selected after fusion. PEG polymers of 1550, 4000 and 6000 mol. wt were found to give very similar numbers of

Table 1. *Effect of PEG molecular weight and concentration, and the time of treatment, on recombination frequencies from protoplast fusion of Streptomyces rimosus mutants 252 and 1054*

The results of three separate experiments are shown.

Factors affecting protoplast fusion			
PEG (Mol. wt)	PEG (%, w/v)	Time of treatment (min)	Frequency of recombination
—	—	1	1.5×10^{-5}
1550	40	1	2.7×10^{-3}
4000	40	1	1.5×10^{-3}
6000	40	1	9.0×10^{-4}
—	—	1	5.3×10^{-5}
1550	20	1	2.5×10^{-4}
1550	40	1	1.3×10^{-3}
1550	60	1	1.0×10^{-3}
1550	40	0	1.1×10^{-5}
1550	40	1	1.1×10^{-3}
1550	40	10	5.3×10^{-3}
1550	40	30	8.0×10^{-2}

regenerating colonies containing recombinants, provided that the concentration (w/v) was the same. The frequencies of recombination dropped, though not significantly, by using PEG of increasing molecular weights. This is consistent with the results obtained by others (Ochi *et al.*, 1979; Baltz & Matsushima, 1981) possibly reflecting the higher viscosity of the solutions tested. The highest recombination frequency was obtained with PEG 1550 at a concentration of 40% (w/v). Below the optimal concentration, the frequency of recombinant production fell drastically by one order of magnitude for each 20% reduction in PEG concentration. Above 40% PEG 1550, recombination frequencies dropped only slightly and may reflect the difficulty of rapidly and uniformly coating the protoplasts with viscous PEG solutions (Hopwood & Wright, 1979). Other authors found that a very brief exposure (about 1 min) to PEG yielded maximal frequencies of recombination (Hopwood & Wright, 1979; Ochi *et al.*, 1979). However, although adhesion between protoplasts of *S. rimosus* mutants occurred immediately on addition of the PEG solution, effective fusions of genomes took longer. The frequency of recombination increased gradually with time, reaching a maximum at 30 min incubation (Table 1). A lower, but significant, number of recombinants obtained after direct selection of protoplasts fused in the absence of PEG-treatment could be ascribed to the presence of Ca^{2+} , which itself promotes fusion (Fodor & Alföldi, 1976).

The recombination frequencies obtained by conjugation and protoplast fusion in crosses of strains 252 and 1054 were 4.0×10^{-5} to 2.2×10^{-4} and 6.4×10^{-2} to 8.7×10^{-2} , respectively (Table 2). Therefore, protoplast fusion of *S. rimosus* mutants allows increase in recombination frequencies by two to three orders of magnitude.

However, the progeny of both conjugation and protoplast fusion consisted of haploid recombinants and about 35% to 50% prototrophic-like heterogenotes (Table 2). Heterogenotes obtained in these experiments showed the ability to grow on all diagnostic media, including minimal agar, when replica-plated. This persisting heterozygous state of many prototrophic-like colonies has been observed previously in crosses of *S. rimosus* mutants. These 'prototrophs' consisted of large, irregular colonies, which segregated both parental and recombinant genotypes (Alačević *et al.*, 1978). In order to test the stability of prototrophic-like heterogenotes, about 100 colonies from both conjugation and protoplast fusion were recloned under non-selective conditions (MM containing growth factors for both parents) and subcultured extensively to encourage the segregation to a stable haploid state. After each subculture, they were replica-plated on minimal agar (MM). Prototrophic-like colonies segregated auxotrophs up to the fourth to fifth subcultures before becoming stable (Table 3). Therefore, heterogenotes persisting through many successive subcultures appeared to be different from the heteroclones described in *S. coelicolor* A3(2) (Sermonti *et al.*, 1960).

Table 2. *Recombination frequencies from crosses by conjugation and by protoplast fusion of Streptomyces rimosus mutants 252 and 1054*

The results of three separate experiments are shown.

No. of colonies tested		Recombinant progeny				Recombination frequency	
		Conjugation		Protoplast fusion			
Con- jugation	Protoplast fusion	No. of re- combinants	No. of heterogenotes	No. of re- combinants	No. of heterogenotes	Con- jugation	Protoplast fusion
213	326	137 (64.3%)	76 (35.7%)	146 (44.8%)	180 (55.2%)	2.2×10^{-4}	7.2×10^{-2}
317	280	196 (61.8%)	121 (38.2%)	135 (48.2%)	145 (51.8%)	8.6×10^{-5}	8.7×10^{-2}
361	339	248 (68.7%)	113 (31.3%)	164 (48.4%)	175 (51.6%)	4.0×10^{-5}	6.4×10^{-2}
Total	891	581 (65.2%)	310 (34.8%)	445 (47.1%)	500 (52.9%)	1.1×10^{-4}	7.4×10^{-2}

Table 3. *Frequency of prototrophic-like colonies persisting after serial restreaking on supplemented non-selective minimal medium*

No. of subcultures	No. of prototrophic-like colonies	
	Conjugation	Protoplast fusion
1	102	104
2	84	93
3	32	40
4	28	27
5	24	27
6	24	27

The appearance of a high frequency of prototrophic-like colonies after fusion of *Streptomyces* protoplasts has been reported (Ochi *et al.*, 1979; Baltz & Matsushima, 1981; Ochi, 1982) and may indicate the persistence of large duplications (Anderson & Roth, 1977) or even of complete chromosomes for many generations. Heterogenotes harbouring abnormal genome size could be employed for the *in vivo* amplification of genes specifically involved in the biosynthesis of an antibiotic. However, genetical or physiological stabilization of duplicated regions would have to be achieved.

Thus, taken together, these results are in striking agreement with those found in other systems (reviewed by Hopwood, 1981) pointing out the importance of 30 min treatment by 40% PEG 1550 to obtain 1–10% recombination by fusion of *S. rimosus* protoplasts. Such recombinant yields are high enough to allow the screening of total non-selected progeny for desirable phenotypes and so eliminating the need for selectable markers. Since the direct selection which was applied allowed the appearance of only 25% of all possible genotypes, these values should be, at least theoretically, multiplied by a factor of four and so yielding recombinant frequencies of up to 40%.

Analysis of six-factor crosses by conjugation and protoplast fusion

The analysis of crosses performed by conjugation and protoplast fusion was done with *S. rimosus* mutants 252 and 1054, each carrying three different mutant alleles. Recombinants were selected for markers on opposite sides on the circular linkage map, with two non-selective markers in each half of the chromosome to divide it into three map intervals of similar distance (Alačević *et al.*, 1973). Such an arrangement of markers was chosen in order to allow us to study the proportions of multiple crossover classes among recombinants, the frequency of the complementary recombinant genotypes, and the acceptability of protoplast fusion technique for long-range mapping.

Table 4. Analysis of recombinant genotypes recovered from crosses by conjugation and by protoplast fusion of *Streptomyces rimosus* mutants 252 and 1054

Genotypes						Crossover intervals	Crossover pattern	Recombinant progeny	
<i>his</i>	<i>pro</i>	<i>nic</i>	<i>rib</i>	<i>pdx</i>	<i>tyr</i>			Conjugation	Protoplast fusion
+	<i>pro</i>	+	+	<i>pdx</i>	+	<i>a, d</i>	2	6	5
+	<i>pro</i>	+	+	+	+	<i>a, e</i>	2	161	48
+	<i>pro</i>	+	+	+	<i>tyr</i>	<i>a, f</i>	2	26	4
+	+	+	+	<i>pdx</i>	+	<i>b, d</i>	2	47	34
+	+	+	+	+	+	<i>b, e</i>	2	81	107
+	+	+	+	+	<i>tyr</i>	<i>b, f</i>	2	84	91
+	+	<i>nic</i>	+	<i>pdx</i>	+	<i>c, d</i>	2	34	23
+	+	<i>nic</i>	+	+	+	<i>c, e</i>	2	83	52
+	+	<i>nic</i>	+	+	<i>tyr</i>	<i>c, f</i>	2	50	20
+	<i>pro</i>	<i>nic</i>	+	<i>pdx</i>	+	<i>a, b, c, d</i>	4	2	16
+	<i>pro</i>	<i>nic</i>	+	+	+	<i>a, b, c, e</i>	4	1	13
+	<i>pro</i>	<i>nic</i>	+	+	<i>tyr</i>	<i>a, b, c, f</i>	4	1	0
+	<i>pro</i>	+	+	<i>pdx</i>	<i>tyr</i>	<i>a, d, e, f</i>	4	1	7
+	+	+	+	<i>pdx</i>	<i>tyr</i>	<i>b, d, e, f</i>	4	1	11
+	+	<i>nic</i>	+	<i>pdx</i>	<i>tyr</i>	<i>c, d, e, f</i>	4	3	12
+	<i>pro</i>	<i>nic</i>	+	<i>pdx</i>	<i>tyr</i>	<i>a, b, c, d, e, f</i>	6	0	2
Total								581	445

Table 5. Analysis of the data in Table 4

No. of crossovers	Recombinants*		No. of possible genotypes	Average no. of recombinants of each possible genotype	
	Conjugation (No.)	Protoplast fusion (No.)		Conjugation	Protoplast fusion
2	572 (98.5)	384 (86.3)	9	63.5	42.7
4	9 (1.5)	59 (13.3)	6	1.5	9.8
6	0 (0.0)	2 (0.4)	1	0.0	2.0
Total	581 (100.0)	445 (100.0)	16	—	—

* Percentage values are given in parentheses.

Recombinants, from the experiments presented in Table 2, were arranged according to the crossovers required to produce them (Table 4). In both conjugation and protoplast fusion, 15 out of 16 possible recombinant classes were recovered. The absence of one of them can be attributed simply to the low number of recombinants analysed. Of the total recombinants arising from six-factor fusion, 86.3% belonged to double crossover classes and 13.3% to multiple crossovers. In contrast, in conventional crosses by conjugation, multiple crossovers represented only 1.5% of the total recombinants (Table 5). Thus, the proportion of multiple crossover classes amongst recombinants was tenfold higher after protoplast fusion than after conjugation.

Crosses of *S. rimosus* mutants have shown that complementary recombinant genotypes have unequal frequencies. Usually one of the two genotypes is ten to one hundred times less frequent than the complementary type (Alačević, 1969). In order to compare the frequencies of complementary genotypes in conjugation and fusion of *S. rimosus* protoplasts, trios of loci were analysed (Table 6). In the example presented, the loci selected were situated in adjacent intervals (*d* and *e*), in intervals separated by one interval (*b* and *d*) and in intervals opposite to each other (*b* and *e*) (Fig. 1). From the results, it appeared that in conjugation the participation of the less frequent genotype was 0.6–25.0% (average 9.0%). In protoplast fusion, the participation of the less frequent complementary genotype doubled (17.9%) and was 1.4–40.2% (Table 6).

Table 6. *Determination of the frequency of complementary recombinant genotypes by the ordering of trios of loci from crosses by conjugation and by protoplast fusion of Streptomyces rimosus mutants 252 and 1054*

Triplet genotypes			Crossover intervals	Crossover pattern	Complementary genotypes*	
					Conjugation (No.)	Protoplast fusion (No.)
<i>pro</i>	+	+	—	—	187 (83.5)	52 (59.8)
+	<i>nic</i>	<i>pdx</i>			37 (16.5)	35 (40.2)
<i>pro</i>	<i>nic</i>	<i>pdx</i>	<i>b</i>	1	2 (1.2)	18 (8.3)
+	+	+			165 (98.8)	198 (91.7)
<i>pro</i>	+	<i>pdx</i>	<i>d</i>	1	7 (5.0)	12 (14.3)
+	<i>nic</i>	+			133 (95.0)	72 (85.7)
<i>pro</i>	<i>nic</i>	+	<i>b, d</i>	2	2 (4.0)	13 (22.4)
+	+	<i>pdx</i>			48 (96.0)	45 (77.6)
<i>pro</i>	+	<i>tyr</i>	—	—	27 (18.8)	11 (12.8)
+	<i>nic</i>	+			117 (81.2)	75 (87.2)
<i>pro</i>	<i>nic</i>	+	<i>b</i>	1	3 (3.4)	29 (22.1)
+	+	<i>tyr</i>			85 (96.6)	102 (77.9)
<i>pro</i>	+	+	<i>e</i>	1	167 (75.9)	53 (62.3)
+	<i>nic</i>	<i>tyr</i>			53 (24.1)	32 (37.7)
<i>pro</i>	<i>nic</i>	<i>tyr</i>	<i>b, e</i>	2	1 (0.8)	2 (1.4)
+	+	+			128 (99.2)	141 (98.6)
<i>pro</i>	+	<i>tyr</i>	—	—	27 (25.0)	4 (6.6)
+	<i>pdx</i>	+			81 (75.0)	57 (93.4)
<i>pro</i>	<i>pdx</i>	+	<i>d</i>	1	8 (5.6)	21 (15.9)
+	+	<i>tyr</i>			134 (94.4)	111 (84.1)
<i>pro</i>	+	+	<i>e</i>	1	162 (97.6)	61 (72.6)
+	<i>pdx</i>	<i>tyr</i>			4 (2.4)	23 (27.4)
<i>pro</i>	<i>pdx</i>	<i>tyr</i>	<i>d, e</i>	2	1 (0.6)	9 (5.4)
+	+	+			164 (99.4)	159 (94.6)

* Percentage values are given in parentheses.

Fig. 1. Arrangement of markers determined by allele frequency gradients in strains 252 (outer circle) and 1054 (inner circle) obtained from crosses (a) by conjugation and (b) by protoplast fusion.

These results are consistent with those obtained by Hopwood & Wright (1978) and reflect nearly complete diploidy of genomes and/or the occurrence of multiple rounds of recombination during or after fusion. The reasons for the somewhat lower expression of the phenomena observed could arise from the incomplete analysis of crosses (the use of only one selective medium), or could be a reflection of cytological differences in the regeneration process itself. That is, the swelling of protoplasts during the regeneration process, reported by Okanishi *et al.* (1974) and Hopwood & Wright (1978), has not been observed in *S. rimosus* (Pigac *et al.*, 1982). However, there are at least three lines of evidence suggesting that the opportunities for crossing over in a fusion of *S. rimosus* protoplasts were spatially and/or temporally greater. These are as follows: (1) electron microscopic examinations of *S. rimosus* protoplasts showed that each contained more than two nuclear equivalents (Pigac *et al.*, 1982); (2) comparison of the DNA contents of *S. rimosus* spores and of protoplasts showed that the latter contained at least twice as much DNA (Ž. Kučan & I. Kučan, unpublished observations) and finally (3) in the course of interrupted conjugation of *S. rimosus* mutants, the first recombinants appeared after 14–16 h incubation of mixed cultures (M. Alačević, unpublished observations). On the contrary, most of the protoplasts regenerated filamentous hyphae after 24–48 h incubation (Pigac *et al.*, 1982). Thus the time for individual genomes to become partitioned into hyphae is at least twice as long in protoplast fusion as in conjugation.

The high frequency of multiple crossover classes obtained after protoplast fusion of *S. rimosus* mutants might have made long-range mapping by this technique ambiguous. Although the map positions of genetic markers used in this study were known, their allele ratios were used to assess their arrangement on the circular linkage map, by minimizing the multiple crossover classes and by calculating allele frequency gradients (Alačević *et al.*, 1973). In protoplast fusion, the average numbers of each genotype were 42·7 for double crossovers, 9·8 for quadruple crossovers and 2·0 for sextuple crossover classes (Table 5). Thus, in recombination through protoplast fusion, each additional requirement for two crossovers reduced the frequency of a genotype by an average factor of 4·5 (42·7/9·8; 9·8/2·0), which is ten times higher than in conventional crosses by conjugation, where the quadruple crossover classes are, at least, 45 (63·7/1·5) times less frequent than double crossovers. However, in spite of that, the data obtained in these experiments reflect linkage relationships around the whole linkage map. This was confirmed by the calculation of allele frequency gradients, which appeared continuous in both conjugation and protoplast fusion (Fig. 1). Therefore, the frequencies of different recombinant classes obtained after protoplast fusion would enable the arrangement of genetic markers on the *S. rimosus* chromosome. This is in agreement with the results obtained in *S. coelicolor* (Hopwood & Wright, 1978) and in *S. fradiae* (Baltz, 1980), where preliminary linkage maps of these species were constructed using the technique of protoplast fusion.

The results presented in this paper show that the availability of a simple, generally applicable procedure to recombine *S. rimosus* strains at high frequency, with a loosening of linkage leading to a nearly random assortment of genotypes in a cross, would greatly facilitate the routine use of recombination in strain improvement.

The authors would like to thank Dr Zora Toman and Dr Vlado Delić for many helpful discussions while critically reading the manuscript. We are also indebted to Mira Debelli and Katarina Pinter for their skilled technical assistance.

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