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Bacterial Protoplast Fusion: Recombination in Fused Protoplasts of *Streptomyces coelicolor*

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Summary. Numerous recombinants arose when protoplasts of *S. coelicolor* were treated with polyethylene glycol and regenerated on non-selective solid medium. In six-factor crosses, recombination frequencies of more than 10% (up to 17%) were routinely observed. This recombination did not require either of the known sex factors, SCP1 and SCP2.

The proportion of multiple crossover classes was much higher than amongst recombinants produced by conjugation between mycelia. Analysis of the spatial distribution of crossovers in double and quadruple crossover recombinants showed only a slight tendency for crossovers to occur closer together than randomly on the complete linkage group. This suggests that genomes brought together by protoplast fusion are complete, or nearly so (in conjugation, in contrast, one genome is represented by a comparatively short fragment). Individual colonies arising from fused protoplasts did not contain different parental genomes without recombinants, but recombinants often occurred without parentals. Several recombinant genotypes often occurred in the same colony, showing a segregation of some, only, of the parental alleles. Complementary genotypes, parental or recombinant, did not occur in the same colony. It is postulated that complete genomes of fused protoplasts usually become fragmented and that crossing-over, often repeated, occurs between the fragments, to generate haploid recombinants.

Analysis of fusions between protoplasts of four different genotypes indicated that the average number of protoplasts fusing together was low, but nevertheless appreciable numbers of fusions involved three or four genomes. Crossing-over between them produced recombinants inheriting markers from three or four parents.

The generation of nearly random populations of recombinants between two or more parent strains by protoplast fusion under the conditions described appears to have simple applications in industrial and academic strain construction.

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Introduction

Artificially prepared bacterial protoplasts have been studied from a physiological and biochemical standpoint since the early 1950's (Marquis and Comer, 1976). However, whereas animal cells, plant protoplasts, and later fungal protoplasts, have been fused for genetical purposes for a number of years (see reviews by Pontecorvo, 1975; Evans and Cocking, 1977; Ferenczy, Kevei and Szegedi, 1976), artificial fusion of bacterial protoplasts is a recent topic of investigation. Fodor and Alföldi (1976) and Schaeffer, Cami and Hotchkiss (1976) described the production of recombinants in protoplast fusions of *Bacillus megaterium* or *Bacillus subtilis*, and Hopwood et al. (1977) found very frequent recombination in fusions within several *Streptomyces* spp.

Conditions for the efficient preparation and regeneration of *Streptomyces* protoplasts have been developed. Sagara et al. (1971) showed that the cell walls are susceptible to rapid digestion by lysozyme if the mycelium is pre-grown in a partially growth-inhibitory concentration of glycine. Okanishi, Suzuki and Umezawa (1974) described procedures for the efficient regeneration of protoplasts on solid media. Their studies were the starting point for our work on protoplast fusion. For the treatment of the protoplasts with polyethylene glycol (PEG), we adopted a procedure closely modelled on one found to be optimal for fusing animal cells (Hales, 1977; Pontecorvo, Riddle and Hales, 1977).

In this paper we describe the genetic consequences of protoplast fusion in *Streptomyces coelicolor* A3(2). We used strains lacking both known sex factors

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(SCP1$^-$ SCP2$^-$) since, in normal mixed cultures of such strains, recombinants arise by mycelial mating at the very low frequency of 10^{-7}–10^{-8} (Bibb, Freeman and Hopwood, 1977). Thus, any confusion of the results of protoplast fusion by recombination occurring between mycelia after wall regeneration was avoided. We set out particularly to answer three questions: (1) are complete genomes from each parent involved in the crossing-over events which follow protoplast fusion? (2) can genomes be involved in more than one round of recombination, as in a bacteriophage cross (Visconti and Delbrück, 1953)? and (3) can more than two protoplasts fuse and their genomes recombine with a significant frequency? The answer to all three questions appears to be affirmative.

Materials and Methods

Bacterial Strains

The genetically marked derivatives of *S. coelicolor* A3(2) are listed in Table 1. All the strains lacked both sex factors, SCP1 and SCP2.

Media and Solutions

The three media, S, P and R2, of Okanishi et al. (1974) were prepared as partial formulations in 80% of their final volume of water and autoclaved. They were then completed, immediately before use, by addition of sterile solutions which had been autoclaved separately (or occasionally Millipore filtered).

Medium S. Glucose, 10 g; Difco Bacto peptone, 4 g; Difco Yeast Extract, 4 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 2 g; K_2HPO_4, 4 g; water to 800 ml. To 20 ml amounts of the sterile medium in 250 ml Erlenmeyer flasks were added appropriate volumes of 20% glycine solution and of sterile water (combined volume 5 ml) to achieve final glycine concentrations of 0.5–4%. For *S. coelicolor* A3(2), 1% was normally used; a higher concentration was needed for most other species (Hopwood et al., 1977).

Medium P. Sucrose, 103 g; K_2SO_4, 0.25 g; trace element solution ($ZnCl_2$, 40 mg; $FeCl_3 \cdot 6H_2O$, 200 mg; $CuCl_2 \cdot 2H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 10 mg; $Na_2B_4O_7 \cdot 10H_2O$, 10 mg; $(NH_4)_6 MO_7O_{24} \cdot 4H_2O$, 10 mg; water, 1000 ml), 2 ml; $MgCl_2 \cdot 6H_2O$, 2.03 g; water to 800 ml. After autoclaving, the following sterile solutions were added to each 80 ml: KH_2PO_4 (0.5%), 1 ml; $CaCl_2 \cdot 2H_2O$ (3.68%), 10 ml; TES (*N*-Tris-(hydroxymethyl)-methyl-2-aminoethanesulphonic acid) buffer (0.25 M, pH 7.2, Millipore filtered), 10 ml.

Medium R2. Sucrose, 103 g; K_2SO_4, 0.25 g; $MgCl_2 \cdot 6H_2O$, 10.12 g; glucose, 10 g; Difco Casaminoacids, 0.1 g; Difco Bacto agar, 22 g; water to 800 ml. After autoclaving, the following sterile solutions were added to each 80 ml: trace element solution, 0.2 ml; KH_2PO_4 (0.5%), 1 ml; $CaCl_2 \cdot 2H_2O$ (3.68%), 8.02 ml; L-proline (20%), 1.5 ml; TES buffer (0.25 M, pH 7.2), 10 ml; NaOH (1N), 0.5 ml.

Lytic Mixture. 1 mg of Lytic Enzyme No. 2 (Kyowa Hakko Kogyo Co., Tokyo, Japan) was agitated vigorously in 10 ml medium P for ca. 5 min to dissolve as much as possible of the poorly soluble powder. 10 mg of lysozyme (Sigma) was then added and the solution was passed through a 0.45 μm Millipore filter.

Concentrated PEG + DMSO. 1.5 ml dimethylsulphoxide (DMSO) was added to 8.5 ml of medium P and the liquid was Millipore filtered. 1.4 ml of this solution was added to 1 g molten PEG 1000 (Koch-Light) previously sterilised by autoclaving to give a concentration of about 40% PEG.

Dilute PEG Solution. 3 ml of medium P was added to 1 g molten sterile PEG 1000 to give a concentration of about 25% PEG.

Table 1. Bacterial strains

John Innes stock number	Genetic markers
2684	*proA1 argA1 uraA1*
2685	*proA1 cysD18 uraA1*
2686	*argA1 cysD18 uraA1*
2692	*proA1 argA1*
M124	*proA1 argA1 cysD18*
M130	*hisA1 uraA1 strA1*
E104	*hisA1 cysD18 uraA1 strA1*

Growth of Mycelium and Protoplast Preparation

Flasks containing 25 ml of medium S plus 1% glycine were supplemented with the growth factors required by the strains, at standard concentrations (Hopwood, 1967). Each flask was inoculated with ca. 10^7 spores and incubated for 3 or 4 d on a shaker at 28–30°C. The rings of growth which were often deposited on the walls of the flasks at the surface of the liquid were suspended in the growth medium once or twice each day to prevent the development of spores which would have been insusceptible to protoplast formation.

The mycelium in one or two flasks of each strain (depending on the amount of growth) was collected by low speed centrifugation for about 5 min, washed with two successive 20 ml volumes of 0.3 M sucrose, and suspended in 1.5 ml of lytic mixture. Care was taken to ensure that no flakes of mycelium remained above the level of the liquid and so escaped the action of the lytic enzymes. After incubation of the mycelium at 30–32°C for 60–90 min, 4 ml of medium P was added and the mycelium was pipetted up and down several times with a 10 ml pipette. The suspension of protoplasts was allowed to pass through a small amount of non-absorbent cotton wool, leaving a considerable deposit of mycelial debris on the cotton wool. Occasionally, the suspension was then filtered through a 2.1 μm Nucleopore filter.

Protoplast Fusion

All operations, up to plating, were done at room temperature (ca. 20°C). Appropriate volumes (half to a fifth of the yield from one or two flasks) of protoplast suspensions of two (or more) strains were mixed. The protoplasts were pelletted by centrifugation at ca. $1000 \times g$ for 8 min, resuspended in 4 ml of medium P, pelletted, resuspended and again pelletted. The pellet was thoroughly drained and dispersed in the minute volume of remaining liquid by gentle rocking. 0.5 ml of concentrated PEG + DMSO was blown

on to the protoplasts and the entire contents of the tube were sucked rapidly in and out of a Pasteur pipette: a flocculent suspension immediately resulted. After 1 min, 0.5 ml of dilute PEG was blown into the suspension and, after a further 3 min, 4 ml of medium P. The suspension was centrifuged at $1000 \times g$ for ca. 5 min and the pellet was gently resuspended in 0.3 ml of medium P for plating. Control preparations, without PEG, were made by suspending the mixed protoplast pellets in 5 ml of medium P, centrifuging, and resuspending in 0.3 ml of medium P.

Genetic Analysis

PEG-treated or control protoplast suspensions were plated undiluted and at dilutions up to 10^{-4} by spreading 0.2 ml with glass rods on R2 medium supplemented with all growth factors required by the parental strains. The plates were incubated for 5–6 days at 30°C.

For mass cultures, spore suspensions were prepared by adding 9 ml of sterile water to each plate and scraping the surface gently with a loop. The suspensions were agitated briefly on a Whirlimixer, filtered through non-absorbent cotton wool, and centrifuged at ca. $1000 \times g$ for 10 min. The spore pellets were suspended in 2 ml of 20% glycerol for immediate plating or storage at −20°C. Serial dilutions (in water or 20% glycerol) were plated on the surface of minimal medium (MM: Hopwood, 1967) containing various combinations of growth factors and sometimes streptomycin to recover parental genotypes, total genotypes, and/or various classes of recombinants. Colonies were counted after incubation at 30°C for 4 d. For further analysis, samples of colonies were picked, 50 per plate, to the same medium and subsequently characterised by replica plating to appropriate diagnostic media.

For single colony analysis, spores from individual colonies arising from 10^{-3} or 10^{-4} dilutions of treated protoplasts on regeneration plates were suspended in a drop of water on a loop and added to 0.2 ml of 20% glycerol. Samples of the resulting suspensions were spotted with a loop on to a series of selective media on which parental or particular classes of recombinant genotypes could grow. Subsequently, suspensions shown to contain recombinants were plated at suitable dilutions on non-selective medium to yield isolated colonies. Samples of these (usually 25 per original colony) were then characterised as above.

DNase Treatment

DNase I (Sigma) was added to the concentrated PEG + DMSO fusion mixture, and to the suspension of fused protoplasts on the regeneration medium, at a concentration of 10 μg/ml.

Results

Lack of Effect of DNase

In our original experiments (Hopwood et al., 1977) we assumed that the observed recombination was due to protoplast fusion rather than to transformation by DNA that might have been released by lysis of a proportion of the protoplasts during the PEG treatment. This conclusion was based on the very high frequencies of recombination and on the inheritance, by many recombinants, of distantly linked markers from both parents. As a direct test of this conclusion, an experiment was done in which DNase was present both during the fusion treatment and during protoplast regeneration. There was no reduction of recombination frequency compared with the control value (Table 2).

Table 2. Lack of effect of DNase on recombination in a fusion of strains M124 and M130

Treatment	Recombinants[a]	
	per minority parent	per majority parent
−PEG	$<1 \times 10^{-6}$	$<2 \times 10^{-7}$
+PEG	3.3×10^{-1}	9.4×10^{-3}
+PEG + DNase	4.9×10^{-1}	1.1×10^{-2}

[a] Selection for *hisA*$^+$ /*strA1*

Table 3. Proportions of recombinants in fusions of strains M124 and M130

Treatment[a]	No. of colonies tested	Recombinants	Percent recombinants
Standard	190	32	16.8
50% PEG (no DMSO)	144	12	8.3
40% PEG (no DMSO)	189	14	7.4
Standard	806	79	9.8
Nucleopore filter	756	116	15.3
Standard	730	46	6.3
Standard	371	62	16.7
Lysozyme only	372	39	10.5
Total (standard treatment)	2097	219	14.4
Total (all data)	3558	400	11.2

[a] Brackets indicate experiments made with the same mycelium or protoplast preparations

Total Analysis of a Six-Factor Fusion Cross

Recombination Frequency. The fusion cross M124 × M130, in which each parent carried a different set of three mutant alleles, was analysed. Spores from confluent cultures of regenerated protoplasts were plated on non-selective medium and the genotypes of samples of the resulting colonies were determined. The frequency of recombinants, for fusions made by the standard procedure, was 6.3–16.8%, with a mean of 14.4% (Table 3). Recombination frequencies in experiments involving some deliberate variations of the standard procedure, while differing slightly from the control values in the same experiments, fell within this range. Lytic Enzyme No. 2, although it resulted in more rapid and complete protoplast formation, was not an essential addition to

lysozyme in the lytic enzyme mixture in order to prepare protoplasts capable of efficient fusion, although further data (not shown) have tended to confirm the indication in Table 3, that recombination frequencies are somewhat higher when lytic Enzyme No. 2 is used. Nucleopore filtration (Okanishi et el., 1974), also, was not essential. The combination of 40% PEG with DMSO appeared to be somewhat better than 40% or 50% PEG without DMSO. In an earlier experiment (Hopwood et al., 1977), in which only selected recombinants were studied, 50% PEG without DMSO appeared to be nearly as good as 40% PEG with DMSO. A more exhaustive study will be needed in order to establish clearly the relationship between recombination frequency and the concentrations of PEG and DMSO.

In our original experiments with *S. coelicolor* A3(2), genetic analysis was done on spores harvested from protoplast mixtures plated *without dilution* on regeneration medium. At that time, no evidence was seen of the inhibition of regenerating protoplast colonies by the minority of colonies developing from non-protoplasted units which we observed with *S. acrimycini* (Hopwood et al., 1977). In later work, such inhibition was sometimes seen. (We did not identify the cause of this altered behaviour, which was probably due to some uncharacterised change in one or more of the medium components.) The result was a reduced recombination frequency amongst spores harvested from denser platings of protoplasts. We therefore began to plate fusion mixtures at a series of tenfold dilutions and to harvest spores from plates on which developing colonies formed a just-confluent culture.

Analysis of Crossing-Over in Mass Cultures. In Table 4 the 400 recombinants from the experiments in Table 3 are arranged, in complementary pairs, according to the crossovers required to produce them. Figure 1 shows the parental arrangement of the six markers, the allele ratio at each locus amongst the recombinants, and the percentage of crossing-over in each of the six map intervals. In Table 4, recombinants requiring two or four crossovers are each sub-divided into three groups according to the relative positions of the individual crossovers. For the double crossover classes, the two individual crossovers may be: in two *adjacent* intervals (e.g. 1 and 2); in two intervals separated by one interval (*intermediate:* e.g. 1 and 3); or in two *opposite* intervals (e.g. 1 and 4). For the quadruple classes, the four individual crossovers may be: in four intervals *all adjacent* to one another (e.g. 1, 2, 3 and 4); in *three adjacent* and one nonadjacent interval (e.g. 1, 2, 3 and 5); or in *two adjacent pairs* of intervals (e.g. 1, 2, 4 and 5).

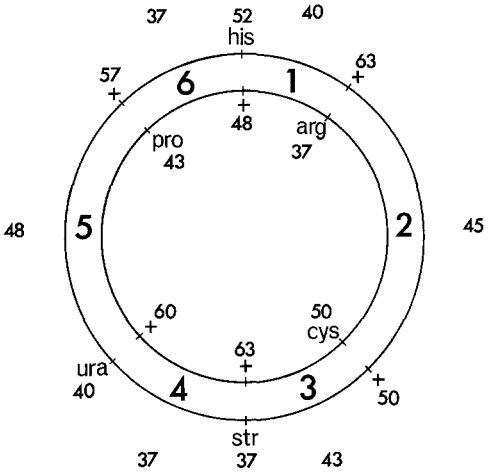

Fig. 1. Arrangement of markers in strains M124 (inner circle) and M130 (outer circle). Allele ratios amongst recombinants, and percentage frequencies of crossing-over (the outer ring of numbers) in each of the map intervals, 1–6 are those in the data in Table 4

Out of 62 possible recombinant genotypes, 57 occurred in the sample, including all 30 double crossover classes, 25 out of 30 quadruple crossover classes and both sextuple crossover classes (Table 4). Of the total recombinants, 76.25% were double crossovers, 23.25% were quadruple crossovers and 0.5% were sextuple crossovers (Table 5). The average number of each genotype was 10.2 for double crossover classes, 3.1 for quadruple crossovers and 1.0 for sextuple crossover genotypes (Table 5); thus each requirement for two crossovers reduced the frequency of a genotype by an average factor of 3.2 (10.2/3.1; 3.1/1.0). The frequencies of crossing-over in each of the six map intervals were quite similar, covering the range 37–48% (Fig. 1); this is much narrower than the range of interval lengths in matings (Hopwood et al., 1973) and reflects the much higher frequencies of recombination. Amongst the recombinants, there was no tendency for alleles from one of the parents to be more abundant than those from the other, the average frequency of the alleles coming from each parent being 50%. However, there were some departures from equality of allele frequencies at particular loci, which were attributable to a reduced viability of several of the mutant alleles; this was most marked for *argA1* and *straA1* (37%), with smaller disadvantages for *uraA1* and *proA1* (Fig. 1).

The numbers of complementary genotypes showed some significant departures from equality. A correction factor was calculated to compensate for the advantage or disadvantage of each allele (e.g. for *argA1*, $50/37 = 1.35$; for $argA^+$ $50/63 = 0.79$) and the number of each genotype was multiplied by the six correction factors appropriate to its constituent alleles. The

Table 4. Analysis of the genotypes of the 400 recombinants in Table 3

Crossover pattern	Crossover intervals[a]	Genotype[b]	Number		Crossover pattern	Crossover intervals[a]	Genotype[b]	Number	
			Actual	Corrected[c]				Actual	Corrected[c]
No c.o. and background	–	pac	1794	–	6 c.o.	1, 2, 3, 4, 5, 6	phau	1	1
		hus	1364	–			cs	1	1
2 c.o. (*adjacent*)	1, 2	pc	21	13	4 c.o. (*all adjacent*)	1, 2, 3, 4	hau	0	0
		haus	6	12			pcs	4	4
	2, 3	pa	13	14		2, 3, 4, 5	pau	2	3
		hcus	18	20			hcs	4	3
	3, 4	hu	12	8		3, 4, 5, 6	phu	6	5
		pacs	10	18			acs	1	1
	4, 5	pacu	10	16		4, 5, 6, 1	phacu	2	3
		hs	20	15			s	4	3
	5, 6	ac	13	11		5, 6, 1, 2	pha	6	6
		phus	7	10			cus	5	6
	6, 1	phac	15	15		6, 1, 2, 3	c	7	3
		us	14	17			phaus	0	0
2 c.o. (*intermediate*)	1, 3	p	12	7	4 c.o. (*3 adjacent*)	1, 2, 3, 5	ha	4	3
		hacus	4	8			pcus	2	3
	2, 4	hcu	14	9		2, 3, 4, 6	au	3	4
		pas	4	7			phcs	1	1
	3, 5	h	27	12		3, 4, 5, 1	pu	10	9
		pacus	5	14			hacs	0	0
	4, 6	acu	4	5		4, 5, 6, 2	phcu	3	3
		phs	2	2			as	5	7
	5, 1	hac	11	8		5, 6, 1, 3	–	4	2
		pus	4	6			phacus	0	0
	6, 2	phc	10	6		6, 1, 2, 4	cu	3	2
		aus	7	15			phas	1	2
2 c.o. (*opposite*)	1, 4	hacu	3	3	4 c.o. (*2 adjacent pairs*)	1, 2, 4, 5	pcu	3	3
		ps	6	6			has	4	5
	2, 5	hc	18	9		2, 3, 5, 6	a	4	3
		paus	3	8			phcus	2	3
	3, 6	ph	7	4		3, 4, 6, 1	u	3	2
		acus	5	10			phacs	0	0

[a] See map in Figure 1

[b] Mutant alleles only indicated: p=*proA1*; h=*hisA1*; a=*argA1*; c=*cysD18*; u=*uraA1*; s=*strA1*

[c] Multiplied by the relative advantage of each allele (see text)

corrected numbers of complementary genotypes turned out to be approximately equal.

The data in Table 5 can be used to consider whether the cellular units in which crossing-over occurs following protoplast fusion are effectively diploid, or whether they contain a complete chromosome from one parent and a chromosome fragment from the other, as is the case in a normal mating between mycelia carrying one or both of the known sex factors (Hopwood, 1967). The relevant data are the proportions of the three arrangements of crossovers amongst the double and amongst the quadruple crossover recombinants; incompleteness of the "zygotes" would be expected to give a great excess of crossovers in adjacent, compared with more separated, intervals. In each case, we expect, on random grounds, a ratio of 2:2:1 for the three arrangements (*adjacent : intermediate : opposite* for the double crossovers; *all adjacent : 3 adjacent : 2 adjacent pairs* for the quadruple crossovers). For the quadruple crossovers,

Table 5. Analysis of the data in Table 4

No. of cross-overs	Progeny		No. of possible genotypes	No. of progeny per genotype	Crossover pattern	No. per crossover pattern
	Number	Percentage				
2	305	76.25	30	10.2	2 c.o. (*adjacent*)	159
					2 c.o. (*intermediate*)	104
					2 c.o. (*opposite*)	42
4	93	23.25	30	3.1	4 c.o. (*all adjacent*)	41
					4 c.o. (*3 adjacent*)	36
					4 c.o. (*2 adjacent pairs*)	16
6	2	0.5	2	1.0	6 c.o.	2

and using the uncorrected genotype frequencies, the observed distribution agrees closely with the random expectation ($\chi^2_2=0.79$, $p=0.65$). For the double crossovers, there is some departure from randomness ($\chi^2_2=19.8$, $p=<0.001$), due primarily to an excess of *adjacent* arrangements. (The calculations were also made on the data after correction for the differential viability of the alleles in each genotype and for the relative frequencies of crossing-over in each map interval; these corrections had a negligible effect on the conclusions – data not shown.) We shall return to the interpretation of these findings in the Discussion.

Analysis of Single Colonies. The procedure was to suspend spores from a series of isolated colonies arising on the regeneration plates after fusion and to make a preliminary assessment of each colony by testing samples of the suspensions (usually containing hundreds or even thousands of spores) on media selecting parental genotypes and genotypes arising by crossing-over in each of several intervals between adjacent loci.

Table 6 gives the results of analysing 98 colonies from each of two crosses with the same six markers in different coupling arrangements. In the first cross, selection could be made for recombinants arising by crossing-over in intervals 1, 4, 5 and 6 (see Fig. 1); in the second cross, in which *cysD18* was re-arranged, the products of crossing-over in each of the six map intervals could be individually selected, thereby decreasing the likelihood of failing to detect colonies containing recombinants. In fact, most colonies yielding recombinants did so on more than one selective medium, so that few colonies containing recombinants would have been missed in the first cross as compared with the second.

Not one colony out of 196 yielded both parental genotypes but no recombinants; this indicated that chance aggregation of protoplasts without true fusion, and protoplast fusion which did not lead to the opportunity for genomic interactions, were rare or absent.

Twentythree of the 196 colonies (12%) yielded identifiable recombinants. These colonies were analysed further by plating the suspensions on non-selective medium and determining the genotypes of samples of 25 of the resulting progeny. In 11 cases, neither parental genotype was found in the sample. Seven out of these 11 colonies yielded only a single recombinant genotype, while four contained several genotypes. In the latter group, loci were evidently heterozygous in the primary fusion colony, but such heterozygosity did not include all six loci. Instead, between one and five loci were heterozygous. All the heterozygous loci were contiguous with each other in three colonies but not in the fourth. The other 12 colonies contained *one* parental genotype, together with recombinants. In seven of the 12 cases the heterozygous loci were all contiguous and in one they were separated by loci that were not heterozygous. In the four remaining colonies, recombinants were rare – none occurred in the sample of 25 progeny analysed – so that the question of contiguity or otherwise of the

Table 6. Analysis of single colonies arising after protoplast fusion

Cross	Parentals only		Recombinants only				Recombinants + a parent			
	One parent	Both parents	One genotype	Multiple genotypes			Contiguous heterozygosity	Non-contiguous heterozygosity	Unknown heterozygosity	Total
				Contiguous heterozygosity	Non-contiguous heterozygosity	Total				
M124 × M130	83	0	5	2	1	8	4	1	2	7
E104 × 2692	90	0	2	1	0	3	3	0	2	5
Total	173	0	7	3	1	11	7	1	4	12

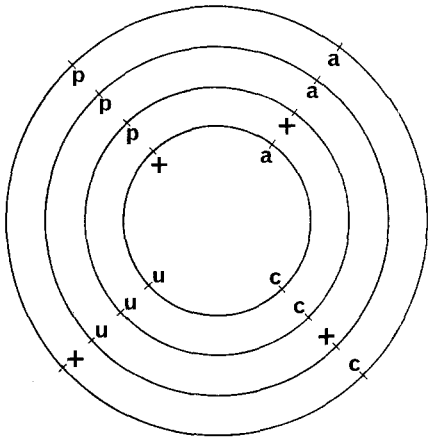

Fig. 2. Arrangement of markers in the four-parent fusion. *p=proA1; a=argA1, c=cysD18; u=uraA1.* Strains (from the outside): M124, 2684, 2685, 2686

heterozygous loci was not answered. An apparent interruption of the sequence of heterozygous loci in the two colonies showing non-contiguous heterozygosity might have been caused by a comparative rarity of certain alleles, which might therefore have been missed in the samples of 25 progeny analysed. However, we failed to find these alleles in a deliberate search amongst hundreds of progeny. Thus, genuine cases existed in which two groups of heterozygous loci were separated by loci that were not heterozygous.

A single colony in which five loci were heterozygous was analysed by the characterisation of 150 colonies. Nine recombinant genotypes occurred in the sample, indicating that several rounds of recombination had taken place. This was not an isolated occurrence: several colonies gave rise to three or four recombinant classes even amongst only 25 progeny examined.

It follows from the absence of some parental alleles from every colony that no colony contained any complementary recombinant genotypes.

A Four-Parent Fusion

Analysis of a Mass Culture. The parental arrangement of markers is in Figure 2. The genotypes were chosen so that each of the four parents carried one prototrophic allele, at a different one of the four loci. This allowed the selective recovery of genotypes arising by crossing-over between different numbers of parental genomes. The fused protoplasts were plated on non-selective regeneration medium and a suspension of spores from the resulting growth was plated on a series of 15 selective media (Table 7). The first four

Table 7. Selective analysis of a four-parent fusion (strains 2684, 2685, 2686, M124), repeated three times (see Fig. 2 for marker arrangement)

Selective medium contains[a]	Cross 1			Cross 2			Cross 3			Average relative frequency	Correction factors[d]			Corrected relative frequency
	Colony count[b]	Total	Relative frequency[c]	Colony count[b]	Total	Relative frequency[c]	Colony count[b]	Total	Relative frequency[c]		Like genome interactions	Crossover probability	Selected genotype abundance	
PAC	51×10^5	275×10^5	1	14×10^5	313×10^5	1	3×10^5	99×10^5	1	1	1	1	1	1
PAU	72×10^5			103×10^5			53×10^5							
PCU	26×10^5			71×10^5			27×10^5							
ACU	126×10^5			125×10^5			16×10^5							
PA	113×10^3	961×10^3	3.6×10^{-2}	41×10^3	513×10^3	1.6×10^{-2}	38×10^3	311×10^3	3.2×10^{-2}	2.8×10^{-2}	4/3	3.2	4	4.8×10^{-1}
PC	548×10^3			259×10^3			21×10^3							
PU	41×10^3			31×10^3			129×10^3							
AC	175×10^3			82×10^3			11×10^3							
AU	27×10^3			45×10^3			73×10^3							
CU	57×10^3			55×10^3			39×10^3							
P	67×10^2	151×10^2	5.9×10^{-4}	142×10^1	829×10^1	2.7×10^{-4}	149×10^1	318×10^1	3.4×10^{-4}	4.0×10^{-4}	8/3	$3.2\sqrt{3.2}$	108	6.6×10^{-1}
A	27×10^2			214×10^1			27×10^1							
C	53×10^2			460×10^1			39×10^1							
U	4×10^2			13×10^1			103×10^1							
—	6×10^0	6×10^0	2.3×10^{-7}	36×10^0	36×10^0	1.2×10^{-6}	0.5×10^0	0.5×10^0	5×10^{-8}	4.9×10^{-7}	32/3	3.2^2	2592	1.4×10^{-1}

[a] P=proline; A=arginine; C=cystine; U=uracil.

[b] The figure is the mean colony count on two plates, multiplied by the dilution factor.

[c] Corrected for the presence of genotypes with fewer auxotrophic requirements amongst colonies on more fully supplemented plates.

[d] See text

media (containing three growth factors) were each capable of recovering one of the parental genotypes; the next six media (containing two growth factors) each recovered a genotype that could arise by crossing-over between two parental genomes; the next four media (containing one growth factor) selected genotypes arising by crossing-over between three parental genomes; and the final medium (unsupplemented) selected a genotype arising from parts of all four parental genomes.

The cross was performed on three occasions. In Table 7, colony counts for media in the same group are summed and expressed relative to the sum of parental genotypes. (In calculating these relative frequencies, small corrections to the colony counts were made to allow for the presence of a minority of genotypes with fewer auxotrophic alleles – e.g. *p* and *a* – amongst those recovered on media allowing the growth of genotypes with more such alleles – e.g. *p a*.) The resulting relative frequencies, which showed satisfactory agreement between the three replicates, were averaged (Table 7).

These raw relative frequencies show that protoplast fusion, followed by crossing-over, can involve more than two parents, since genotypes occur in which prototrophic alleles have come from three or four different parents. Selected genotypes arising from the interaction of more than two parental genomes are rare (4.0×10^{-4} for three parents and 4.9×10^{-7} for four). However, these frequencies give a very misleading picture of the proportions of fusions involving different numbers of protoplasts. The system is too complex to obtain accurate estimates of these proportions. However, by making some simplifying assumptions we can derive several correction factors which allow the calculation of rough estimates of the relative proportions of colonies arising from one, two, three or four protoplasts.

One correction allows for the varying probabilities that protoplasts fusing in groups of two, three or four will be genetically different and therefore capable of generating recombinants. For groups of two, 3/4 cases will involve unlike genomes (giving a correction factor of 4/3); for groups of three, the proportion is 3/8 (correction factor 8/3); and for groups of four it is 3/32 (correction factor 32/3). (This assumes equal proportions of each parental genotype, a condition that varies from cross to cross.)

A second correction allows for the requirement of varying numbers of crossovers in order to generate the selected genotypes: the doubly auxotrophic genotypes each require *two* crossovers between two different parental genomes; the single auxotrophic genotypes each require *three* crossovers involving three different genomes; and the prototrophic genotype needs *four* crossovers involving four different genomes. Approximate correction factors come from the results of the two-parent cross analysed earlier. There was a ratio of 3.2 between the frequencies of double versus quadruple and quadruple versus sextuple crossover genotypes; the correction factor is therefore approximately $\sqrt{3.2}$ for every successive crossover.

A third set of correction factors allows for the fact that the selected genotypes represent varying proportions of the total outcome of the appropriate numbers of crossovers between different numbers of parental genomes. To derive them we consider the number of ways in which the various crossover patterns can occur and the fraction of the total outcome which the selected genotypes represent. For the interaction between any two different genomes (for example *pac* and *pau*), there are six patterns of double crossing-over (the six ways of choosing two intervals out of four), giving 12 products, and three of these are the selectable genotype (*pa*); thus the correction factor is 4. For interactions between three different genomes (for example *pac, pau* and *pcu*), a crossover in any interval can involve any two of the three genomes, giving three possibilities. Triple crossing-over, in any three intervals, therefore occurs in 3^3 different ways. There are four combinations of three intervals; therefore the number of possible configurations of three crossovers is $3^3 \times 4 = 108$. Each configuration gives two products, but two of the total yield of products represent the selectable genotype (*p*); therefore the correction factor is 108. For interactions between all four different genomes, a crossover in any interval, involving any two of the four genomes, can occur in six ways. There are therefore 6^4 patterns of quadruple crossing-over, and each gives two products. Thus there are $6^4 \times 2 = 2592$ products of quadruple crossing-over. Only one of these is the selected, prototrophic, genotype, so the correction factor is 2592.

When these correction factors are applied (Table 7), the relative probabilities of interactions involving 1, 2, 3 and 4 parents are of the order of 100:48:66:14 (44:21:29:6%). There are numerous approximations involved in arriving at these estimates. Therefore it would be inappropriate to put too much weight on their precise values. However they do establish that a considerable proportion of interactions involves more than two parental genomes: nevertheless the average number of protoplasts developing into each colony, derived from the estimates, is only about 2.

Analysis of Single Colonies. Spore suspensions from 100 isolated colonies from the four-parent fusion were tested on the four media each allowing the growth

Table 8. Analysis of single colonies from a four-parent fusion (strains 2684, 2685, 2686, M124). See Figure 2 for marker arrangement

Number of parents involved	Growth on selective media	Single colonies	
		Number	Total
1	PAC	19	58
	PAU	17	
	PCU	11	
	ACU	11	
2	PAC, PAU	6	25
	PAC, PCU	4	
	PAC, ACU	1	
	PAU, PCU	4	
	PAU, ACU	6	
	PCU, ACU	4	
3	PAC, PAU, PCU	1	7
	PAC, PAU, ACU	4	
	PAC, PCU, ACU	2	
	PAU, PCU, ACU	0	
4	PAC, PAU, PCU, ACU	0	0
2, 3 or 4	PACU only	10	10

of one parental genotype. Spores from 58 colonies grew on one, only, of the selective media: the numbers of the four parental types were rather similar (Table 8), so that this cross was well "balanced". Spores from 25 colonies grew on two selective media and those from seven grew on three. Spores from the remaining ten colonies were exclusively of the quadruple auxotrophic genotype.

The spore suspensions which grew on two or three of the parental media were re-tested on a set of ten media, containing the six combinations of two growth factors and the four single growth factors. Each of the 25 suspensions which had given growth on two parental media produced colonies on the medium containing the two growth factors common to these two media. Each of the seven suspensions which had grown on three parental media grew on the medium containing the single growth factor in common to them (and on the doubly supplemented media containing this growth factor). Therefore, as in the case of the two-parent fusion, there was no example in which two (or three) parental genotypes were present in the same colony without the selectable genotype which could arise by recombination between both (or all three) of them. Again, appreciable numbers of colonies contained recombinants only – in this cross the ten colonies containing only the quadruple auxotrophic genotype.

As in the mass analysis, the proportions of colonies coming from more than one protoplast are underestimated because those involving like genotypes go undetected. The first set of correction factors used in Table 7 can be applied, but the class of colonies containing exclusively the quadruple auxotrophic genotype is difficult to assign because it could represent interactions between two, three or four genomes. If we ignore this class, the percentage of groups of 1, 2, 3 and 4 protoplasts are of the order of $48:27:16:\leqq 9$, giving an average number of protoplasts per group of about 2; satisfyingly, the same value as was derived from the mass analysis.

Discussion

These data confirm our earlier conclusion from experiments in which only certain classes of recombinants were selected from the spores produced on regenerated protoplast cultures (Hopwood et al., 1977) that very high frequencies of recombination occur when *Streptomyces* protoplasts are induced to fuse with PEG, even in the absence of known sex factors. In a six-factor cross analysed without selection, overt recombinants represented up to 17% of the progeny and would doubtless have been found to be even more frequent of higher numbers of markers had been used.

The starting point for the present experiments was the earlier observation, confirmed in the present study, that the proportions of multiple crossover classes amongst recombinants was higher, by a factor of more than ten, after protoplast fusion than after mating in a mycelial culture. We speculated that this might be due to a more complete diploidy in the cellular units produced by protoplast fusion than in the merozygotes arising by mating, and/or to the occurrence of multiple rounds of recombination following protoplast fusion (Hopwood et al., 1977). We felt the latter to be a plausible explanation in view of the discovery by Okanishi et al. (1974) that protoplasts of the streptomycetes that they studied swelled to a large size (some 20 times the original diameter) during the regeneration process; they might therefore come to contain several genomes which could interact repeatedly, as in a bacteriophage cross (Visconti and Delbrück, 1953). In *S. coelicolor*, under our conditions, such swelling also occurred (I. Stevenson and D.A. Hopwood, unpublished observations) although to a lesser extent (about five diameters).

The finding of several recombinant genotypes in many of the single colonies arising from a two-parent fusion showed that repeated opportunities for recombination often occur (in the early stages of regeneration and growth of the colony since, by the time spores are harvested from the colony, they behave

as pure haploids). However, a single recombination event, presumably before any genome replication, is also a common occurrence since an appreciable proportion of colonies consisted of a single recombinant genotype. A striking and unexpected finding was that no colony analysed contained all the parental alleles, either in the form of both parental genotypes, or as a pair of complementary recombinants.

At first sight, this finding might suggest that the process of protoplast fusion typically produces an incompletely diploid cellular unit. However, analysis of the distribution, between map intervals, of the crossovers required to produce the observed progeny suggests that the *primary* fusion cells are diploid, or nearly so. The evidence is that, in both the double and the quadruple crossover classes, there were high proportions of progeny arising from crossovers widely separated on the chromosome. Amongst the quadruple crossover genotypes, the spatial distribution of the constituent crossovers was approximately random, with very little tendency for adjacent arrangements to be in excess. Amongst the double crossover genotypes, there was a statistically significant excess of adjacent doubles but nevertheless crossovers in diametricaly opposite intervals of the linkage map represented as many as 14% of double crossovers, compared with the 20% expected on random grounds.

In order to explain the observations we suggest that protoplast fusion does indeed bring complete genomes from each of the participating protoplasts into the same cellular unit but that one, and often both, genomes become fragmented, with the loss of some segments. Recombination then occurs, often repeatedly. If a complete genome survives, recombination between it and one or more genome fragments can give a colony containing one parental class and one or more recombinant classes; alternatively, if both parental genomes become fragmented, one or more recombinant classes occur without a parental genotype. It may even be that fragmentation of both parental genomes is the rule, since a parental genotype could still occur if all of its alleles were retained on one or more genome fragments. There was a marked tendency for the region of heterozygosity in each colony to include a set of contiguous loci – this occurred in ten out of 12 cases that could be analysed – rather than to be interrupted by loci that were not heterozygous. This observation suggests that recombination events typically involve small numbers of comparatively long genome segments. (The finding that many colonies contained a series of different genotypes arising by recombination of a group of contiguous markers would account for the statistical excess of the adjacent double crossover progeny in the analysis of the mass culture.)

The probability of crossing-over between genomes brought together by protoplast fusion must be very high, approaching 100%. This conclusion arises from a failure to find any single colonies containing different parental genotypes but no recombinants, either in the two-parent or in the four-parent fusion. It is confirmed by the finding that the average total proportion of recombinants in mass cultures of regenerating protoplasts from a two-parent fusion (11.2%; Table 3) was about the same as the proportion of single colonies containing any recombinants (12%; Table 6 – this is a slight underestimate since occasionally a colony might have contained a recombinant class that was not detected). In further agreement with the same conclusion is the fact that broadly similar estimates for the proportions of fusions involving different numbers of genomes in the four-parent fusion were obtained from the mass culture and from the single colony analysis. If the probability of recombination had been much below 100%, the single colony analysis would have indicated much higher proportions of fusions in all except the single genome class.

Are our conclusions about the genetic consequences of protoplast fusion in *Streptomyces* likely to reflect special features of these mycelial bacteria, or are they applicable also to eubacteria? In the two published studies on bacilli, the questions posed in our work were not asked so directly, partly because colonies derived from regenerating fused protoplasts of auxotrophic strains were always recovered on media selecting for prototrophy for some or all of the markers. However, there are enough resemblances, and no important qualitative differences, between the results obtained with *Bacillus* and *Streptomyces* to suggest that very similar events take place. In *B. subtilis*, Schaeffer et al. (1976) found high proportions of quadruple and sextuple crossover recombinants in six-factor crosses involving markers widely spread over the genome, indicating extensive, if not complete, diploidy in the cellular units produced by protoplast fusion. Individual colonies were not examined in this study. In *B. megaterium*, Fodor and Alföldi (1976) found that individual colonies recovered on selective media after protoplast fusion gave rise to various genotypes of auxotrophic cells on sub-culture. In both the bacilli, as in *S. coelicolor*, heterozygosity after protoplast fusion was evidently transient (although not confined to a single genomic interaction) and no constantly segregating clones were detected. If conditions for efficient protoplast fusion can be developed in Gram-negative species, it will be very interesting to see if similar phenomena occur as in the Gram-positive streptomycetes and bacilli.

The four-parent fusion in *S. coelicolor* allowed an estimate to be made of the average number of protoplasts involved in each fusion event. The low number obtained (about 2) confirms the distinction, pointed

out but not quantified in *B. subtilis* by Schaeffer et al. (1976), between the *aggregation* of protoplasts into large clumps visible to the naked eye, which occurs immediately on exposure to PEG, and *fusion* itself: the aggregates largely disperse on dilution of the concentrated PEG solution while protoplasts presumably remain attached at effective fusion sites which have already arisen between small numbers of the protoplasts within each aggregate. That fusion itself, like aggregation, occurs very rapidly has been demonstrated for animal cells by Pontecorvo et al. (1977).

We emphasized in our earlier paper (Hopwood et al., 1977) the possible application of protoplast fusion to strain improvement in *Streptomyces* by allowing the production, without introducing counterselective markers into the parental strains, of populations containing high proportions of recombinants between them. The present results, which indicate that multiple crossover events, which occur frequently between genomes, involve long regions of the genomes, rather than being confined to short segments, confirm the potential utility of protoplast fusion in generating all possible combinations of the genes of two parent strains; the effects of linkage are very much reduced as compared with the normal mating process. Furthermore, it turns out that recombination between the genomes of three, or even four, parents in a protoplast fusion are not rare. These multi-parent fusions may provide a useful mode of operation when a series of divergent lines are available, carrying alleles which may interact in a beneficial way to produce a desirable phenotype.

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