Protoplast Fusion in *Streptomyces*: Fusions Involving Ultraviolet-irradiated Protoplasts

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Protoplasts of *Streptomyces coelicolor* showed the same ultraviolet killing kinetics as spores. Irradiated protoplasts gave rise to recombinants when they were fused with unirradiated protoplasts of a strain carrying complementary genetic markers. The decline with u.v. fluence in the capacity of irradiated protoplasts to yield recombinants inheriting individual markers was some six times less steep than that of the survival of unfused protoplasts; thus, for example, protoplasts reduced to only 0.01% survival still yielded 10% as many recombinants as unirradiated protoplasts. Each of six widely separated markers of the irradiated parent was inherited independently of the others, with a frequency falling exponentially with u.v. fluence.

INTRODUCTION

The discovery that protoplasts of several species of *Streptomyces* can be fused in the presence of polyethylene glycol and regenerated to yield high proportions of recombinants for chromosomal genes (Hopwood *et al.*, 1977; Hopwood & Wright, 1978, 1979; Baltz, 1978; Ochi & Katz, 1978; Ochi *et al.*, 1979; Hopwood, 1981*b*) has considerable implications for both the academic and applied genetics of these important antibiotic-producing organisms (Hopwood & Chater, 1980; Hopwood, 1981*a, c*). In a number of species, the proportion of recombinants amongst the total spores produced on a regenerating fused culture, or the proportion of individual regenerating colonies containing recombinants, was so high – up to at least 20% under optimal conditions – that it was quite feasible to analyse fusion crosses, or isolate particular desired recombinant genotypes from them, without having any counter-selectable marker in each parent. However, in some species, the proportion of recombinants may be much lower than 20%, even in intra-strain fusions, making the use of selective methods imperative. This may occur either because fusion conditions are sub-optimal (Hopwood, 1981*b*) or because of the presence of a significant proportion of non-protoplasted units (which yield non-recombinant progeny: Hopwood *et al.*, 1977). In such situations, and others, ultraviolet (u.v.) irradiation of both parental protoplast preparations immediately before fusion may significantly increase the proportion of recombinants, presumably by allowing the selective survival of recombinants arising by patterns of crossing-over which eliminate lethal u.v. hits at different positions in the parental genomes (Hopwood & Wright, 1979). This approach might also be useful in inter-strain or inter-specific fusions in which, for reasons which may include incomplete genome homology, the presence of restriction-modification systems, or physiological incompatibility between two strains (Hopwood, 1981*b*), recombinants may be quite rare (Godfrey *et al.*, 1978).

Here we describe experiments in which only one of the parental protoplast preparations is treated with u.v., before fusion with unirradiated protoplasts of the other. Applications of this technique may include cases where counter-selectable markers are available in one strain but not in others with which it is to be recombined, a situation likely to hold when protoplast fusion is used for the discovery of new antibiotics by recombination (Hopwood, 1981*a, d*); or where limited regions of the genome of one strain are to be introduced into a second strain without disturbing the rest of its genotype.

Table 1. *Strains of Streptomyces coelicolor*

Strain no.	Markers					
	<i>proA1</i>	<i>hisA1</i>	<i>argA1</i>	<i>cysD18</i>	<i>uraA1</i>	<i>strA1</i>
2708	—	—	—	—	—	S
2709	—	—	—	—	—	R
2805	+	+	+	+	+	R
2806	—	+	—	—	—	S
2872	—	—	—	+	+	S
2873	+	+	+	—	—	S
M124	—	+	—	—	+	S
M130	+	—	+	+	—	R
M145	+	+	+	+	+	S

Fig. 1. Arrangement of the markers on the *S. coelicolor* linkage map.

METHODS

Bacterial strains. These were derivatives of *Streptomyces coelicolor* A3(2) from the John Innes Institute culture collection (Table 1). They all lacked both sex factors, SCP1 and SCP2, thus eliminating any confusion of the results by recombination through mating between mycelia on the regeneration plates, since recombinants occur in SCP1⁺SCP2⁺ × SCP1⁺SCP2⁺ crosses at frequencies not greater than 10⁻⁷ (Bibb *et al.*, 1977). The positions of the markers on the linkage map are shown in Fig. 1.

Protoplast preparation and fusion. The 'standard' conditions of Hopwood & Wright (1978) were used with the following modifications: lytic enzyme no. 2 was usually omitted from the lytic mixture; fusion was promoted by treatment of protoplast pellets with 1 ml 50% (w/v) polyethylene glycol (PEG) 1000 for 2 min, followed by dilution with 4 ml medium P, centrifugation and plating on a non-selective medium consisting of R2 agar containing growth factors required by both parents. Three plates were prepared, carrying, respectively, undiluted samples of the fusion mixture and samples diluted 10-fold and 100-fold. After incubation of the plates for 5–7 d, spores were harvested from one or more of the plates with confluent, or nearly confluent, well-sporulating colonies, but not from any plates on which very dense growth resulted in a retardation of sporulation by precociously-developing non-protoplasted units (Hopwood & Wright, 1979). The spore suspensions were plated on a series of media consisting of MM (Hopwood, 1967) containing combinations of growth factors selective for particular genotypes. Colonies were counted after 4 d incubation.

Ultraviolet irradiation. This was performed as described by Hopwood & Wright (1979) at a dose rate of about 0.83 J m⁻² s⁻¹.

RESULTS

Ultraviolet sensitivity of protoplasts and spores

Figure 2 shows that the sensitivity of protoplasts to killing by u.v. irradiation, in the absence of subsequent PEG treatment, was not significantly different from that of spores (Harold & Hopwood, 1970*a*).

Table 2. *Effect of ultraviolet irradiation of one parent on the frequency of recombinants after protoplast fusion*

Parent strains		U.v. fluence (J m ⁻²)	Recombinants*	Recombinants after irradiation relative to unirradiated control
Irradiated	Unirradiated		Unirradiated parent†	
—	M130, M124	0	8.4×10^{-2}	10^0
M130	M124	500	7.3×10^{-3}	8.7×10^{-2}
M124	M130	500	3.5×10^{-3}	4.2×10^{-2}
—	M145, 2708	0	5.7×10^{-2}	10^0
M145	2708	500	3.9×10^{-3}	6.8×10^{-2}
M145	2708	1000	6.6×10^{-4}	1.2×10^{-2}
—	2873, 2872	0	6.7×10^{-2}	10^0
2873	2872	250	2.5×10^{-2}	3.7×10^{-1}
2873	2872	500	4.1×10^{-3}	6.1×10^{-2}
2873	2872	1000	1.3×10^{-3}	1.9×10^{-2}
—	2873, 2872	0	2.8×10^{-1}	10^0
2873	2872	1000	3.9×10^{-3}	1.4×10^{-2}
2873	2872	1500	1.0×10^{-3}	3.6×10^{-3}
2873	2872	2000	5.6×10^{-4}	2.0×10^{-3}

* Average frequency of colonies on media selecting recombinants inheriting single markers from the irradiated parent.

† Frequency of colonies on medium selecting the genotype of the unirradiated parent.

Fig. 2

Fig. 3

Fig. 2. Relationship between u.v. fluence and survival of spores or protoplasts, or frequency of recombinants for single markers from the irradiated parent after fusing irradiated protoplasts with unirradiated protoplasts: ●, survival of spores (average values for experiments with strains 2805 and M145); ○, survival of protoplasts (average values for strains 2805, M145, 2709 and 2873); ▲, frequency of recombinants, relative to unirradiated control (data of Table 2).

Fig. 3. Relationship between u.v. fluence and frequency of recombinants inheriting one, two or three markers from the irradiated ('donor') parent as a fraction of the progeny having the genotype of the unirradiated parent. The parents were strain 2873 (irradiated) and strain 2872 (unirradiated). Recombinants were selected to contain *cysD*⁺*uraA*⁺ (from the unirradiated parent) together with the following markers from the irradiated parent: ○, *argA*⁺; □, *hisA*⁺; ▽, *proA*⁺; ●, *hisA*⁺*argA*⁺; ■, *proA*⁺*argA*⁺; ▼, *proA*⁺*hisA*⁺; ▲, *proA*⁺*hisA*⁺*argA*⁺.

Table 3. *Frequencies of colonies arising on various selective media from a fusion of unirradiated protoplasts of strain 2806 with irradiated protoplasts of strain 2805*

The u.v. fluence was 500 J m^{-2} . The fused protoplasts were regenerated on non-selective medium and the resulting spores were replated on a series of 16 media each selecting a different combination of markers from the irradiated parent.

No. of markers from irradiated parent	Markers from irradiated parent	No.* of colonies (count $\times$ dilution)	Average no.
0	None	170×10^5	1.70×10^7
1	<i>pro</i> ⁺ <i>arg</i> ⁺ <i>cys</i> ⁺ <i>ura</i> ⁺	96×10^3 124×10^3 70×10^3 168×10^3	1.15×10^5
2	<i>pro</i> ⁺ <i>arg</i> ⁺ <i>arg</i> ⁺ <i>cys</i> ⁺ <i>cys</i> ⁺ <i>ura</i> ⁺ <i>ura</i> ⁺ <i>pro</i> ⁺ <i>pro</i> ⁺ <i>cys</i> ⁺ <i>arg</i> ⁺ <i>ura</i> ⁺	183×10^2 172×10^2 110×10^2 168×10^2 132×10^2 162×10^2	1.55×10^4
3	<i>pro</i> ⁺ <i>arg</i> ⁺ <i>cys</i> ⁺ <i>arg</i> ⁺ <i>cys</i> ⁺ <i>ura</i> ⁺ <i>cys</i> ⁺ <i>ura</i> ⁺ <i>pro</i> ⁺ <i>ura</i> ⁺ <i>pro</i> ⁺ <i>arg</i> ⁺	268×10^1 224×10^1 164×10^1 271×10^1	2.32×10^3
4	<i>pro</i> ⁺ <i>arg</i> ⁺ <i>cys</i> ⁺ <i>ura</i> ⁺	413×10^0	4.13×10^2

* Average of two plates.

Effect of ultraviolet irradiation on the capacity of protoplasts to produce recombinants

Protoplasts of several strains were irradiated for different periods, fused with unirradiated protoplasts carrying complementary markers, and regenerated to produce sporulating cultures. Samples of these spores were plated on a medium recovering the phenotype of the unirradiated parent and on a series of two to five media each selecting a class of recombinants differing from the unirradiated parent by having one marker from the irradiated parent. The averages of the colony counts on the media selecting recombinants are given as fractions of the parental counts (Table 2). The frequency of recombinants after u.v. irradiation for each fluence, expressed relative to the control value without irradiation, gave average estimates of the recombination frequency for each fluence (Table 2); these are plotted as a function of u.v. fluence in Fig. 2. The rate of decline with u.v. fluence of the capacity of irradiated protoplasts to yield recombinants was much lower than the loss of viability of unfused protoplasts. For example, at 1% survival, the recombinant frequency was only reduced to 25% of the control level, while at 0.01% survival, recombinants were still nearly 10% as frequent as without irradiation.

Independent inheritance of markers from irradiated protoplasts

In a further series of experiments, spores produced on regenerated cultures arising from fusions between irradiated and unirradiated protoplasts were plated on a set of media selecting recombinants inheriting one, two, three or four markers (chosen from *hisA*⁺, *proA*⁺, *argA*⁺, *cysD*⁺ and *uraA*⁺ in various crosses) from the irradiated parent. An example of the resulting colony analysis is given in Table 3. Differences between the frequencies of inheritance of the various pairs or trios of markers were no greater than those of the individual markers; therefore, in the analysis of the data, the particular group of markers in each experiment, and the sequence of the markers on the linkage map, appear to be

Fig. 4. Numbers of progeny with different numbers of markers of the irradiated parent for various u.v. fluences. Fusions involved the following pairs of strains ('donor' markers in parentheses): ●, 2873 (irradiated) $\times$ 2872 (*proA*⁺ *hisA*⁺ *argA*⁺); ▼, M130 (irradiated) $\times$ M124 (*proA*⁺ *argA*⁺ *cysD*⁺); ▲, 2805 (irradiated) $\times$ 2806 (*proA*⁺ *argA*⁺ *cysD*⁺ *uraA*⁺); ■, M145 (irradiated) $\times$ M124 (*proA*⁺ *argA*⁺ *cysD*⁺); ♦, M145 (irradiated) $\times$ 2708 (*proA*⁺ *hisA*⁺ *argA*⁺ *cysD*⁺ *uraA*⁺).

Fig. 5. Relationship between u.v. fluence and the proportion of recombinants inheriting each 'donor' marker (from the slopes of the curves in Fig. 4).

unimportant. The data were considered in two ways to investigate the effect of irradiation on the probability of inheritance of groups of markers from the irradiated parent (the 'donor').

In Fig. 3, the frequencies of recombinants inheriting one, two or three donor markers are plotted as a function of u.v. fluence. Curves for the three individual markers are similar, as are those for the three pairs of markers. However, the sets of curves (for one, two or three markers) have markedly different slopes, indicating a strong effect of irradiation in reducing the joint inheritance of groups of genes from the irradiated parent. This is confirmed by Fig. 4, which shows the numbers of recombinants plotted against the number of donor markers which they contain, for each u.v. fluence. The proportion of recombinants inheriting each successive donor marker was essentially constant for a given u.v. fluence, since the curves (in this semi-logarithmic plot) approximate to straight lines with slopes characteristic of the

fluence. The average values for these slopes (that is the proportion of those recombinants inheriting n donor markers which also inherit $n + 1$ markers) are: 7.4×10^{-1} without irradiation; 3.0×10^{-1} at 250 J m^{-2} ; 1.5×10^{-1} at 500 J m^{-2} ; and 2.8×10^{-2} at 1000 J m^{-2} . When these values were plotted against u.v. fluence, the exponential relationship in Fig. 5 was obtained.

DISCUSSION

The main objective of these experiments was to see if protoplasts irradiated to a negligible survival level could yield viable recombinants when fused with unirradiated protoplasts. This turned out to be strikingly true. The method could therefore be used to eliminate any parental strain(s) devoid of conventional counter-selective markers in fusions with marked strains, as an alternative to heat inactivation of one strain (Fodor *et al.*, 1978) or chemical killing of one or both strains by protein inhibitors of different specificity (Wright, 1978), which have been described for *Bacillus* protoplasts or mammalian cells as methods to enrich for recombinants. Clearly the genetic consequences of u.v. irradiation, acting primarily on DNA, should be different from those of inactivation by heat or by the chemical denaturation of proteins; whereas the latter procedures might not bias the genotype of recombinants in favour of one parent or the other, u.v. would be expected to do so, and this was clearly demonstrated in these experiments.

The finding that unfused protoplasts show an identical u.v. killing curve to that of spores (Fig. 2) suggests that protoplasts, like spores, are effectively haploid and that repair processes after irradiation (Harold & Hopwood, 1970*a, b*) operate similarly in both situations. The fluence for 37% survival over the exponential region of the curve was 40 J m^{-2} in these experiments. The yield of viable recombinants by irradiated protoplasts when fused with unirradiated protoplasts fell much more slowly with irradiation, the 37% fluence being about 250 J m^{-2} over the exponential region. Evidently, segments of the genome of the irradiated parent, free of lethal hits, could be dissociated by crossing-over from lethal damage elsewhere in the genome and incorporated into viable recombinants. The ratio of target sizes implied by the different mean lethal fluences for protoplast survival and recombinant production (250/40) suggested that the genomes of recombinants (viable or inviable according to u.v. fluence) which inherit a marker from the irradiated parent consist, typically, of about one-sixth of the genome of the irradiated parent (the 'donor') and about five-sixths of that of the unirradiated parent (the 'recipient').

The five markers used here, although clearly linked in conjugative matings (Hopwood *et al.*, 1973), showed only loose linkage in fusions of unirradiated protoplasts, because of the greater opportunities for recombination provided by the presence of complete parental genomes in fused protoplasts and the occurrence of several successive rounds of recombination before or during regeneration (Hopwood & Wright, 1978). Since a mean lethal fluence of 40 J m^{-2} for unfused protoplasts implies the production of one lethal hit per average-sized map interval between five markers per 200 J m^{-2} , it is not surprising that linkage between the markers of the irradiated parent was rapidly destroyed by irradiation, being virtually undetectable even at the lowest u.v. fluence of 250 J m^{-2} studied here. Each marker of the irradiated parent was inherited by recombinants independently, with a probability exponentially related to fluence (Fig. 4). In other words, as irradiation increases, any selected marker inherited by viable recombinants from the irradiated parent will be surrounded by a progressively shorter adjacent segment of the genome of that parent, and will also contain progressively less numerous and less extensive unlinked segments of its genome. With high fluence, a particular short region of the genome of a recipient strain, in the vicinity of a counter-selectable marker, could be replaced by a corresponding donor segment, with a minimum of other genomic substitutions. Of course, non-lethal mutations in the 'target' region would tend to be inherited, and this might be turned to advantage in order to 'focus'

mutagenesis in a particular region. To this end, we could contemplate massive mutagenesis of the donor population with an efficient general mutagen (such as nitrosoguanidine: Delić *et al.*, 1970), followed by u.v. irradiation, fusion of protoplasts with the recipient strain, and selection for an appropriate donor marker.

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