Genetic Mapping in *Streptomyces clavuligerus* by Protoplast Fusion

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The development of a protoplast manipulation protocol for the industrially important bacterium *Streptomyces clavuligerus*, which produces the β -lactamase inhibitor clavulanic acid, made possible a preliminary genetic mapping study based on protoplast fusion crosses. A preliminary position for 11 markers on the *S. clavuligerus* genetic map is proposed. Fusion progeny were characterized by random spore analysis because the markers present in the strains were not amenable to the conventional four-on-four selection procedure. Whilst the resulting map is similar to that derived by conjugation for *S. clavuligerus* and *S. coelicolor*, further analysis of the markers is required to confirm these observations.

INTRODUCTION

Recombination in streptomycetes was first demonstrated in *Streptomyces coelicolor* (Sermonti & Spada-Sermonti, 1955). However, it was the introduction of protoplast fusion methods (Hopwood *et al.*, 1977; Baltz, 1978) that provided the impetus for genetic crossing in a broader range of these organisms, in which the application of conjugation had not been feasible, e.g. *S. fradiae* (Baltz, 1980) and *Nocardia lactamdurans* (Wesseling & Lago, 1981). Knowledge of the genetic map locations of antibiotic genes should be useful for interspecific hybrid formation, for directed mutagenesis using selectable markers closely linked to the antibiotic structural and regulatory genes, and for gene amplification by classical and recombinant DNA techniques (Queener & Baltz, 1979; Hopwood & Chater, 1984).

Genetic recombination between auxotrophically marked strains of *S. clavuligerus* (ATCC 27064) following conjugation has been described (Aharonowitz & Demain, 1977), and subsequently a genetic map of fourteen markers was established (Kirby, 1978). A series of protoplast fusion crosses with *S. clavuligerus* was made in this laboratory primarily to identify suitable model crosses to study the enhancement of recombination (Illing, 1987); however, the resulting data permitted the preliminary location, reported here, of eleven new genetic markers on a chromosomal map. The validity of this map needs confirmation using conjugation crosses.

METHODS

Isolation of mutants. Auxotrophic and drug-resistant mutants were isolated mainly using UV irradiation. A few were obtained following *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or ethyl methanesulphonate treatment (Illing, 1987). The sandwich layer technique (Saunders *et al.*, 1982) was used to isolate drug-resistant mutants that could not be recovered by direct selection. Mutants with altered sulphur utilization were isolated using the method of Arst (1968).

Protoplast fusion. Protoplasts were isolated by the method described in the accompanying paper (Illing *et al.*, 1989). Equal volumes (10 ml) of the protoplast suspension (1×10^7 or 1×10^8 protoplasts ml⁻¹) were mixed and

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centrifuged (2000 g, 10 min) and the pellet was resuspended in the small volume of medium P + BSA (Illing *et al.*, 1989) remaining when the supernatant was removed. Then 1 ml medium P (Illing *et al.*, 1989), without BSA, containing 33% (w/v) polyethylene glycol was gently added to the protoplasts and the suspension was carefully mixed. After 2 min, 4 ml medium P without BSA was added and the protoplasts were recovered by centrifugation. The protoplasts were washed in medium P, and finally resuspended in 1 ml of this solution. The suspension was serially diluted and 100 µl samples of the dilutions with 10^8 and 10^7 protoplasts ml⁻¹ were inoculated onto solid R6 medium (Illing *et al.*, 1989), supplemented with the specific requirements of the strains. Control platings of the fusion mixture and the individual protoplast suspensions included 100 µl of a 10^{-1} dilution in Tween 80 inoculated on R6, 100 µl of a 10^{-1} dilution inoculated on MSD⁺2 [MSD⁺ (Bailey *et al.*, 1984), with the concentrations of yeast extract and bacteriological peptone doubled to 4 and 5 g l⁻¹, respectively] and 100 µl of a mixture of protoplasts, untreated with PEG, inoculated on R6.

The cultures were incubated at 26 °C until no further colonies appeared (7–21 d). The mycelium was scraped into sterile distilled water, diluted and plated on MSD⁻R (Illing *et al.*, 1989), plus supplements, to obtain sporulation. In some experiments, benlate (50 µg ml⁻¹) was used to prevent fungal contamination. Sporulation occurred after 7–14 d, and these cultures were used to prepare spore suspensions in sterile distilled water. The suspensions were filtered through glass wool to remove mycelial fragments. Spores were also resuspended in 20% (w/v) sucrose and maintained at -20 °C.

The genotypes of progeny derived from crosses were identified by the analysis of randomly isolated spores; suspensions were plated on MSD⁺2 at dilutions to give single colonies. Colonies were isolated to prepare master plates which could be replicated on to diagnostic media for characterization. The data were analysed using the Genetics.p or GENETICS computer programs (Illing & Onions, 1986; Illing, 1987). The frequency of recombination was calculated from the ratio of the number of progeny in a recombinant class to the total number of progeny tested.

RESULTS

Protoplast fusion in S. clavuligerus

Fourteen protoplast fusion crosses were made (Table 1). Because of leakiness with some of the genetic markers in the strains used, it was necessary to recover and analyse between 200 and 400 random spore isolates. These crosses generated recombination data for the 11 mutations involved, but it was not possible to score the *argA1* marker because it was too leaky. The markers *cysA14*, *cysA44*, *cysA46* and *cysA48* were found to be allelic as were *thrA2* and *thrA8*. The data were analysed in a pair-wise fashion and used to establish a preliminary linkage map. Where only single values for recombination frequency are available the relative positions of markers must be regarded as tentative.

A sample of 260 random isolates was tested for reversion at each mutant site; no reversion was found. A more exhaustive analysis for reversion could only be carried out with strains which had tight mutations, i.e. 12-8, 12-19, 12-132 and 12-143, and in each case the reversion frequency was less than 1 in 10^7 .

Construction of a preliminary genetic map

The data were used to assign the eleven genetic markers to preliminary positions on a *S. clavuligerus* genetic map (Fig. 1). A summary of the recombination data is given in Table 2. Greatest significance was placed on the crosses which exhibited allele ratios which most closely approached unity (Table 3). Divergence from unity may reflect different capacities of protoplast regeneration, sporulation exhibited by the strains or differential selective pressure against the different markers.

The markers on the map are positioned at equal distances from each other since recombination data from protoplast fusion crosses do not accurately assign distances on a linkage map (Keller *et al.*, 1983, 1985; Hopwood *et al.*, 1985).

The cross between 12-132 (*proA1 acrA6*) and 12-143 (*hypA1 cysA46*), which was repeated several times, provided a basis for the map with an order (clockwise) of *proA1*, *acrA6*, *hypA1 cysA46*. Further markers could then be positioned on the map by a systematic analysis of additional recombination data for these and other markers.

proA cysA; *proA cysB*; *cysA cysB*. The recombination frequencies between *proA* and *cysA* varied greatly. A notable feature was that combination of *cysA* and *cysB* in a cross appeared to

Fig. 1. Preliminary genetic map of *S. clavuligerus*.

Table 1. Protoplast fusion crosses

Cross	Strain	Genotype*	Strain	Genotype*
C1	12-124	(<i>proA1 cysB6</i>)	×	12-143 (<i>hypA1 cysA46</i>)
C2	12-125	(<i>proA1 rifA1</i>)	×	12-143 (<i>hypA1 cysA46</i>)
C3	12-132	(<i>proA1 acrA6</i>)	×	12-143 (<i>hypA1 cysA46</i>)
C4	12-124	(<i>proA1 cysB6</i>)	×	12-143 (<i>hypA1 cysA46</i>)
C6	12-124	(<i>proA1 cysB6</i>)	×	12-141 (<i>hypA1 cysA44</i>)
C7	12-125	(<i>proA1 rifA1</i>)	×	12-141 (<i>hypA1 cysA44</i>)
C9	12-52	(<i>pheA1 cysA14</i>)	×	12-124 (<i>proA1 cysB6</i>)
C10	12-52	(<i>pheA1 cysA14</i>)	×	12-132 (<i>proA1 acrA6</i>)
C14	12-132	(<i>proA1 acrA6</i>)	×	12-143 (<i>hypA1 cysA46</i>)
CF1	12-14	(<i>argA1 pheA1</i>)	×	12-74 (<i>proA1 metA4</i>)
CF2	12-14	(<i>argA1 pheA1</i>)	×	12-75 (<i>proA1 leuA1</i>)
CF4	12-14	(<i>argA1 pheA1</i>)	×	12-77 (<i>proA1 thrA2</i>)
CF5	12-23	(<i>argA1 metA4</i>)	×	12-75 (<i>proA1 leuA1</i>)
CF6	12-91	(<i>proA1 thrA8</i>)	×	12-151 (<i>lysA1 cysA48</i>)

* Auxotrophic requirements: *arg*, arginine; *cys*, cysteine; *hyp*, hypoxanthine; *leu*, leucine; *lys*, lysine; *met*, methionine; *phe*, phenylalanine; *pro*, proline; *thr*, threonine. Inhibitor resistance: *acr*, acriflavine; *rif*, rifampicin.

depress the overall recombination frequency exhibited. Additionally, the *cysA cysB* recombinant colonies could not be recognized using a simple plate test. It has been shown that *cys* mutations reduce protoplast regeneration frequencies (Illing, 1987) and possibly sporulation, thus affecting the recovery or detection of *cysA cysB* recombinants. Excluding the recombination values from crosses combining both these markers significantly reduced the variation and gave a median recombination frequency for *proA* and *cysA/B* of 15.9%. This figure was on the boundary of tight linkage suggested by Hopwood (1972) and indicated that the *lysA* and *pheA* markers were located between them, *lysA* being closer to *proA* than *pheA*. Where *cysA* and *cysB* were analysed in the same cross they appeared to be very tightly linked and inseparable at the level of recombination detected. The exception was cross C9, which indicated that *pheA* maps closer to *cysA* than it does to *cysB*; this may reflect the different genetic background for *cysA*. The same order was not confirmed by linkage to *proA* but the allele ratios of *proA* in this cross exhibited the greatest departure from unity, possibly explaining this discrepancy.

hypA cysA; *hypA cysB*. Recombination frequencies indicated that *cysA* and *cysB* mapped closer to the *hypA* marker than *proA*.

hypA proA. Most of the crosses indicated that *proA* and *hypA* were unlinked. Although in some cases the data for *proA-cysA* and *proA-hypA* may suggest that *hypA* was closer to *proA* than *cysA*, when the results from each cross were considered this hypothesis was not substantiated.

Recombination frequency (%)

Cross		Recombination frequency (%)													
Markers	no. . . .	C1	C2	C3	C4	C6	C7	C9	C10	C14	CF1	CF2	CF4	CF5	CF6
<i>proA</i>	<i>cysA</i>	1.2	18.9	28.1	0.8	0.8	20.8	14.7	10.0	11.2	—	—	—	—	15.9
<i>cysA</i>	<i>cysB</i>	0	—	—	0	0	—	3.9	—	—	—	—	—	—	—
<i>hypA</i>	<i>cysA</i>	6.5	4.6	3.5	9.6	8.1	4.6	—	—	2.7	—	—	—	—	—
<i>hypA</i>	<i>proA</i>	5.4	15.1	4.6	8.9	7.3	19.2	—	—	10.8	—	—	—	—	—
<i>hypA</i>	<i>cysB</i>	6.5	—	—	9.6	8.1	—	—	—	—	—	—	—	—	—
<i>cysB</i>	<i>proA</i>	1.2	—	—	0.8	0.8	—	11.6	—	—	—	—	—	—	—
<i>proA</i>	<i>rifA</i>	—	14.3	—	—	—	18.9	—	—	—	—	—	—	—	—
<i>cysA</i>	<i>rifA</i>	—	10.8	—	—	—	6.5	—	—	—	—	—	—	—	—
<i>hypA</i>	<i>rifA</i>	—	3.9	—	—	—	8.9	—	—	—	—	—	—	—	—
<i>proA</i>	<i>acrA</i>	—	—	7.3	—	—	—	—	11.2	13.9	—	—	—	—	—
<i>cysA</i>	<i>acrA</i>	—	—	3.1	—	—	—	—	3.5	8.1	—	—	—	—	—
<i>hypA</i>	<i>acrA</i>	—	—	3.5	—	—	—	—	—	7.7	—	—	—	—	—
<i>proA</i>	<i>pheA</i>	—	—	—	—	—	—	8.5	8.5	—	14.4	4.5	20.1	—	—
<i>cysA</i>	<i>pheA</i>	—	—	—	—	—	—	10.0	3.1	—	—	—	—	—	—
<i>cysB</i>	<i>pheA</i>	—	—	—	—	—	—	13.1	—	—	—	—	—	—	—
<i>acrA</i>	<i>pheA</i>	—	—	—	—	—	—	—	4.2	—	—	—	—	—	—
<i>proA</i>	<i>metA</i>	—	—	—	—	—	—	—	—	—	9.5	—	—	0.8	—
<i>pheA</i>	<i>metA</i>	—	—	—	—	—	—	—	—	—	19.3	—	—	—	—
<i>proA</i>	<i>leuA</i>	—	—	—	—	—	—	—	—	—	—	4.1	—	3.9	—
<i>pheA</i>	<i>leuA</i>	—	—	—	—	—	—	—	—	—	—	6.2	—	—	—
<i>proA</i>	<i>thrA</i>	—	—	—	—	—	—	—	—	—	—	—	8.9	—	13.5
<i>pheA</i>	<i>thrA</i>	—	—	—	—	—	—	—	—	—	—	—	16.0	—	—
<i>metA</i>	<i>leuA</i>	—	—	—	—	—	—	—	—	—	—	—	—	3.1	—
<i>cysA</i>	<i>thrA</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	21.6
<i>cysA</i>	<i>lysA</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	7.2
<i>lysA</i>	<i>proA</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	11.7
<i>lysA</i>	<i>thrA</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	20.5
All classes		6.5	20.4	9.2	9.6	8.1	24.6	17.0	12.7	16.9	21.6	7.4	23.5	3.9	25.8

Number of progeny

[illegible]

The order (anti-clockwise) of *proA cysA hypA* was also confirmed by recombination data for *cysA* and *hypA* and for *acrA* and *rifA*.

proA rifA; *cysA rifA*; *hypA rifA*. Crosses C2 and C7 were used to order these markers. The former gave allele ratios closest to unity and was favoured where discrepancies arose. Of these three markers (*proA*, *cysA*, *hypA*) *proA* exhibited the lowest degree of linkage to *rifA* and in cross C7 the two markers appeared to be unlinked. The order indicated by cross C2 was (anti-clockwise) *cysA hypA rifA*. In both crosses *cysA* exhibited tighter linkage to *hypA* than to *rifA*.

proA acrA; *cysA acrA*; *hypA acrA*. Results from the three crosses (C3, C10, C14) suggest two possible orders, with the *acrA6* and *hypA1* markers in reversed positions. However, based on allele ratios which most closely approached unity the data support an anti-clockwise order *proA1 cysA46 hypA1 acrA6*. Other data (Illing, 1987) favoured this orientation in a ratio of 7:1. The ordering of markers *acrA* and *rifA* was done by considering crosses C2 and C14 (fusions with the best allele ratios for the markers concerned) and the two following orders are proposed (anti-clockwise order): (a) *cysA hypA acrA rifA proA*; (b) *cysA hypA rifA acrA proA*. Configuration (b) fitted the data most accurately with only one discrepancy, whereas (a) had three. Further crosses would be required to confirm this order.

proA pheA; *cysA pheA*; *cysB pheA*. Using the data from cross C9 *pheA* was placed between *proA* and *cysA*. The results from this cross suggested that *cysB* mapped further away from *pheA* than did the *cysA* marker. This was not consistent with the order with respect to *proA* but this marker in this cross exhibited the most aberrant allele ratio.

acrA pheA. The data from cross C10 appeared to show linkage between *pheA* and *acrA*. Significance was not attached to this result because the allele ratios were extremely aberrant, possibly due to sporulation problems with strain 12-52.

proA metA, and other combinations. The order (moving clockwise) *proA metA leuA thrA* was suggested for the following reasons. Assuming the validity of the preliminary order (moving clockwise) *lysA pheA proA*, based on cross CF6 in which *proA* mapped closer to *pheA* than did *lysA*, the order derived from cross CF2, *pheA proA leuA*, was orientated to give the overall order *lysA pheA proA leuA*. Using the results of cross CF5, i.e. *proA metA leuA*, to build on this order, the map was extended to: *lysA pheA proA metA leuA*.

Finally, the *thrA* locus was placed between *leuA* and *acrA* because the recombination frequencies for *proA* and *thrA* (8.9%, 13.5%) were greater than those for *proA* and *leuA* (3.9%, 4.1%) but less than those for *proA* and *acrA* (13.9%). The data showed that *pheA*, *cysA* and *lysA*, all proposed on the opposite side of the linkage map to *thrA*, were unlinked to *thrA*. The 3 o'clock region of the map (particularly) requires further crosses to fully validate the order of *leuA thrA acrA rifA*.

The allele ratios associated with the crosses used in the construction of the preliminary genetic map of *S. clavuligerus* are given in Table 3. Where aberrant allele ratios were observed this was largely due to the unequal recovery of the parental strains. Thus in cross C10 the ratio of 226:1 was probably due to the low recovery of strain 12-52 arising from its poor sporulation.

In the crosses where sufficient data were obtained to draw firm conclusions, none of the markers exhibited a marked selective advantage or disadvantage thereby dramatically affecting their frequency of occurrence in the progeny of the cross. Therefore, to prevent aberrant recombination frequencies distorting the order of the markers on the map, crosses exhibiting allele ratios closest to unity were used. The allele ratios of most auxotrophic markers showed a slight bias towards prototrophy, e.g. *cysA* (+:–1.41:1), *hypA* (+:–1.51:1), *proA* (+:–1.37:1). Recombination frequencies between particular markers were markedly perturbed by one fusion, i.e. cross C10, from which aberrant allele ratios were obtained. This cross highlighted how combinations of disadvantageous auxotrophic markers gave a much greater overall deleterious effect than the two markers acting independently. The drug-resistance marker *acrA* was shown to be slightly advantageous (*acrA* +:–1:1.28).

DISCUSSION

The procedure adopted for the analysis of the first set of fusion crosses was dictated by the fact that the strains were not amenable to standard four-on-four analysis (Hopwood *et al.*, 1985). In some cases colonies developed from spores inoculated onto selective media, indicating that certain markers were leaky. This caused difficulty in their characterization by the direct selection method, a problem also observed in *S. rimosus* (Friend & Hopwood, 1971).

Two hundred and sixty isolates were considered to be a sufficiently large sample for analysis. The randomly selected recombinants were retested to confirm results. Back mutation of all the genes analysed was significantly lower than the recombination frequencies observed and was not a factor in the crosses described. Regenerants derived from untreated mixtures of parental protoplasts did not include recombinants. No selective pressure was applied against the parental strains in the recovery of the cross progeny. Thus their number complicated the analysis of the data in the pair-wise data tables.

Protoplast fusion has been shown to distort genetic maps because of the ensuing participation of whole chromosomes and the consequent increase in the level of recombination possible, leading to loosened linkage and near-random assortment of genotypes in some crosses (Hopwood *et al.*, 1977; Pigac *et al.*, 1982; Hranueli *et al.*, 1983; Keller *et al.*, 1983; Hopwood & Chater, 1984). However, this loosened genetic linkage does not itself produce ambiguity in the cases analysed, e.g. *S. coelicolor* (Hopwood & Wright, 1978), *S. lividans* (Hopwood *et al.*, 1983) and *S. rimosus* (Hranueli *et al.*, 1983).

A genetic map of *S. clavuligerus* has been previously established by a series of conjugative crosses (Kirby, 1978) and is similar to that presented here. As the strains used in producing the earlier map were not available to us, complementation studies could not be carried out and therefore it is not possible to discuss the basis of the differences between the two linkage maps and the significance of conjugation and protoplast fusion as mechanisms for recombination in *S. clavuligerus*. Despite insufficient information on the complementary nature of phenotypically similar mutations in *S. coelicolor* (Hopwood *et al.*, 1985) and *S. clavuligerus* there are some similarities between the maps of the two species. When more data are available one would expect to observe similarities of gene distribution on the two chromosomal maps, a phenomenon observed with other streptomycetes (Rhodes, 1986).

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