Genetic Analysis in *Streptomyces chrysomallus*

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A circular linkage map was developed for *Streptomyces chrysomallus*, a producer of actinomycin C. The map order of various marker loci was deduced from matings and to a minor extent from protoplast fusions. The map strongly resembles that of *Streptomyces coelicolor* A3(2). The recombination frequencies were low and highly variable (from 10^{-9} to 5×10^{-6}). Plasmid pIJ303 expressed its thiostrepton resistance gene in *S. chrysomallus* but did not promote chromosomal transfer or induce the Ltz^+ phenotype. The data provide a background of genetics for investigations of antibiotic synthesis in this strain.

INTRODUCTION

Streptomyces chrysomallus produces actinomycins, bicyclic chromopeptide lactone antibiotics, the synthesis of which is widespread among actinomycetes (Katz, 1967). Recent biochemical studies with *S. chrysomallus* and *Streptomyces antibioticus* revealed that 4-methyl-3-hydroxyanthraniloyl pentapeptide lactones are the immediate precursors of actinomycins. In addition, a 4-methyl-3-hydroxyanthranilic acid (4-MHA) activating enzyme, most probably involved in actinomycin synthesis, could be isolated from *S. chrysomallus* (Keller, 1984; Keller *et al.*, 1984). These results, and current biochemical work on pentapeptide lactone synthesis in this laboratory, make *S. chrysomallus* an obvious candidate for genetic studies on the synthesis of these antibiotics. Such genetic work may involve the characterization of mutants with blocks in the synthetic pathway by complementation analyses and screening for specific enzyme defects. In addition, mapping studies could add information about the number of genes involved and also about their location on the chromosome, as has been shown in the cases of actinorhodin and undecylprodigiosin synthesis in *Streptomyces coelicolor* A3(2) (Rudd & Hopwood, 1979, 1980). As a prerequisite for such studies a background of genetics must be available. In this paper we describe the construction of a circular linkage group of *S. chrysomallus* with a limited number of marker loci.

METHODS

Media. Complete medium (CM) for maintenance of strains and for mutant isolation was a modification of that of Pridham *et al.* (1956/57). It contained (per litre): 5 g malt extract (Wander, Bern, Switzerland), 5 g yeast extract (Difco), 5 g maltose (Serva, Heidelberg, FRG) and 15 g agar (Oxoid no. 3), and was adjusted to pH 7.5 with 1 M-NaOH.

Minimal medium (MM) was the same as that described by Hopwood (1967), except that glucose was substituted by 0.5% (w/v) maltose. Maltose was autoclaved separately as a 25% (w/v) solution and added to the medium before cooling. Nutritional requirements were added in concentrations as described previously (Friend & Hopwood, 1971). Regeneration medium for protoplast fusion cultures was based on R2 medium and contained 0.25 M-sucrose (Keller *et al.*, 1983). Since many of the strains used regenerated with frequencies of less than 0.1%, fusion cultures were routinely regenerated in the additional presence of 0.5% yeast extract (Thompson *et al.*, 1980), which increased the regeneration frequencies of many strains by a factor of 5–100. The same medium was also used for the regeneration of protoplasts transformed with plasmid DNA. Soft regeneration agar for the selection of

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Abbreviation: MM, minimal medium.

Table 1. *Auxotrophic markers*

Marker	Requirements	Marker	Requirements
<i>ade-1</i>	Adenine or guanine	<i>pab-1</i>	<i>p</i> -Aminobenzoic acid
<i>arg-1</i>	Arginine, citrulline or ornithine (non-allelic with <i>arg-8</i>)	<i>pdx-1</i>	Pyridoxine
<i>arg-2</i>	Arginine	<i>phe-1</i>	Phenylalanine
<i>arg-8</i>	Arginine, citrulline or ornithine	<i>pro-1</i>	Proline
<i>cys-1</i>	Cysteine	<i>pro-2</i>	Proline
<i>his-1</i>	Histidine or histidinol	<i>ser-1</i>	Serine
<i>ile-1</i>	Isoleucine + valine	<i>thr-3</i>	Threonine
<i>leu-1</i>	Leucine	<i>trp-2</i>	Tryptophan
<i>met-4</i>	Methionine or homocysteine	<i>ura-1</i>	Uracil

transformants contained thiostrepton ($25 \mu\text{g ml}^{-1}$ final concentration) or neomycin ($20 \mu\text{g ml}^{-1}$) and was a mixture of the regeneration medium and protoplast buffer P medium (Okanishi *et al.*, 1974) in a ratio of 1:1.

Strains. All strains of *S. chrysomallus*, bearing up to five auxotrophic markers, were derived from strain ATCC 11523. They were maintained on CM slants or kept as spore suspensions in 30% (v/v) glycerol at -20°C .

Mutagenic treatments. Mutant strains were isolated after treatment of spore suspensions either with UV light at 254 nm or by exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as described previously (Delić *et al.*, 1970). Mutagenesis with ethylmethane sulphonate (EMS) was effective when spores were suspended in 30% glycerol containing 5 mg ml^{-1} of the mutagen.

Genetic markers. Treatment of *S. chrysomallus* spores with any of the three mutagens yielded only a limited number of auxotrophic phenotypes; the characteristics of the auxotrophic markers used in this study are shown in Table 1. Since the viability of mutants very often decreased from one mutagenic step to the next, nearly all strains bearing more than two markers were constructed by recombination.

Crosses. Generally, strains to be crossed differed from each other by at least two characters. Before crossing, the strains were tested for their genotype and reversion rate. Each strain was streaked separately on four or five slants ($25 \times 150 \text{ mm}$) of appropriately supplemented MM. After growth for 4–6 d at $28\text{--}30^\circ\text{C}$, 6 ml sterile water was added to each slant and the agar surface was carefully scraped into the water with a loop. The resulting suspensions of spores and mycelial clumps were poured into screw-cap vials and vortexed vigorously. After filtration through cotton wool the spore suspensions of each strain were combined and centrifuged (5000 g for 15 min). The pelleted spores were resuspended in 2–3 ml sterile water. Portions of the spore suspensions from the two parental strains were mixed in an approximate ratio of 1:1 (estimated by optical density at 650 nm) and 0.1–0.2 ml of the mixed suspension was inoculated onto each of 16–24 slants of fully supplemented MM (i.e. with all parental requirements). Alternatively, in the case of poorly sporulating strains, Petri dishes with the same medium were used. The large number of slants or plates in each cross was necessary in order to obtain enough spores; *S. chrysomallus* does not sporulate well and has a rather low fertility.

Crosses by protoplast fusion were performed as described previously (Keller *et al.*, 1983). Because of the much higher recombination frequencies compared to those of matings, one or two regeneration plates were sufficient for recovery of large numbers of recombinants.

Isolation and analysis of recombinants. Cross slants or plates were incubated for 6 d at $28\text{--}30^\circ\text{C}$ and spores were isolated as described above. The purified and combined spores were suspended in 2–3 ml water ($10^9\text{--}10^{11}$ c.f.u.) and tenfold dilutions were made up to 10^{-11} . Samples (0.1 ml) from dilutions $10^{-1}\text{--}10^{-4}$ were plated in duplicate on plates containing selective media, and from dilutions $10^{-4}\text{--}10^{-11}$ on parental media. Undiluted samples (0.1 ml) of the original suspension were also plated on five plates of each selective medium because the fertility of crosses could be very low (see Results). The plates were incubated for 3 d and colonies on selective media were transferred to plates of the same medium with sterile toothpicks. After incubation for 1 week these master plates were replica plated onto diagnostic media for genotype identification. Heterokaryons among these samples could easily be recognized by their failure to grow on the same selective medium from which they had been isolated; they usually represented less than 10% of the total non-parental colonies, and their frequencies depended on the length of the selected interval (see Results). Successful crosses usually yielded 200–4000 recombinants per cross, representing recombination frequencies of between 10^{-9} and 5×10^{-6} .

Introduction of plasmid DNA into S. chrysomallus by transformation. The plasmids pIJ303 and pIJ211 (kindly provided by Professor D. A. Hopwood, Norwich, UK) were introduced into several genetically marked strains of *S. chrysomallus*. Isolation of pIJ303 and pIJ211 from their *Streptomyces lividans* hosts, PEG-mediated transformation, and selection of transformants was done essentially as described by Chater *et al.* (1982).

Table 2. Analysis of a four-factor cross of *S. chrysomallus* strains E1 (*arg-8 ser-1*) and R24 (*leu-1 phe-1*)

The four-factor cross was done following the procedure of Hopwood (1972).

Genotypes of selectable progeny	Frequencies of genotypes recovered on selective media supplemented with:				Mean frequency of each pair of complementary genotypes
	Arg + Leu	Arg + Phe	Ser + Leu	Ser + Phe	
+ + + +	89	94	85	78	86.5
<i>arg</i> + + +	11	10	—	—	10.5
+ <i>leu</i> + +	92	—	57	—	74.5
+ + <i>ser</i> +	—	—	26	18	22
+ + + <i>phe</i>	—	5	—	4	4.5
<i>arg leu</i> + +	8	—	—	—	4.5
+ + <i>ser phe</i>	—	—	—	1	
<i>arg</i> + + <i>phe</i>	—	1	—	—	1.5
+ <i>leu ser</i> +	—	—	2	—	
Total no. of recombinants per plate	200	110	170	101	
No. of recombinants examined	250	249	249	250	

Relative recombination frequency in each interval (including the corresponding values from Tables 3 and 4):

	<i>arg-8-leu-1</i>	<i>arg-8-phe-1</i>	<i>ser-1-leu-1</i>	<i>ser-1-phe-1</i>	<i>arg-8-ser-1</i>	<i>leu-1-phe-1</i>
Table 2	117.5	184.5	103.0	176.0	38.5	85.0
Table 3	28	84	17	89	13	88
Table 4	62.0	179.5	58.5	177.0	16.5	203.5

RESULTS

Mapping of four marker-loci by four-factor crosses

Establishing a genetic map of a new streptomycete is commonly initiated by determining the arrangement of four markers in four-factor crosses by the procedure of Hopwood (1972). In such crosses, spores from mixed cultures of two double auxotrophs are seeded onto four selective media, each containing two requirements in non-parental combinations. By genotype analysis of haploid recombinants recovered from the selective media, it is possible to deduce the arrangement of the markers involved. Tables 2, 3 and 4 show the results of three sets of four-factor crosses between double auxotrophs of *S. chrysomallus*, involving the mutations *arg-8*, *leu-1*, *phe-1* and *ser-1*. In each cross these four markers were present in different parental combinations. This was possible because all four doubly auxotrophic complementary recombinant genotypes were recovered from the first cross (Table 2). In each cross the frequencies of recombinants of a particular genotype recovered on the four different media did not differ greatly from each other, and this also applied to the frequencies of the members of the two pairs of complementary genotypes. These are prerequisites for the analysis. From the numbers of classes of complementary genotypes on the different media, the average frequencies could be calculated (final column in Tables 2, 3 and 4). From these average frequencies, the relative recombination frequencies between the six pairs of markers in each cross was derived (lower part of Table 2).

In all three crosses the frequency of recombination in the *arg-8-ser-1* interval had the lowest value, whereas the intervals *arg-8-phe-1* and *ser-1-phe-1* had high values. The intervals *ser-1-leu-1* and *arg-8-leu-1* showed intermediate values. The interval *leu-1-phe-1* had high values in Tables 3 and 4. From the relative recombination frequencies, two possible marker arrangements could be deduced, as shown in Fig. 1. Following the procedure of Baumann *et al.* (1974) the

Table 3. *Analysis of a four-factor cross of S. chrysomallus strains R74 (arg-8 phe-1) and R75 (leu-1 ser-1)*

Genotypes of selectable progeny	Frequencies of genotypes recovered on selective media supplemented with:				Mean frequency of each pair of complementary genotypes
	Arg + Leu	Arg + Ser	Phe + Leu	Phe + Ser	
+ + + +	10	7	6	9	8
<i>arg</i> + + +	75	69	—	—	72
+ <i>leu</i> + +	1	—	1	—	1
+ + <i>phe</i> +	—	—	3	5	4
+ + + <i>ser</i>	—	8	—	8	8
<i>arg leu</i> + +	8	—	—	8	8
+ + <i>phe ser</i>	—	—	—	—	
<i>arg</i> + + <i>ser</i>	—	0	—	—	0
+ <i>leu phe</i> +	—	—	0	—	
Total no. of recombinants per plate	94	84	10	30	
No. of recombinants examined	250	248	246	245	

Table 4. *Analysis of a four-factor cross of S. chrysomallus strains R73 (arg-8 leu-1) and R76 (phe-1 ser-1)*

Genotypes of selectable progeny	Frequencies of genotypes recovered on selective media supplemented with:				Mean frequency of each pair of complementary genotypes
	Arg + Phe	Arg + Ser	Leu + Phe	Leu + Ser	
+ + + +	3	9	3	6	5
<i>arg</i> + + +	47	28	—	—	37.5
+ <i>phe</i> + +	1	—	2	—	1.5
+ + <i>leu</i> +	—	—	4	5	4.5
+ + + <i>ser</i>	—	150	—	161	155.5
<i>arg phe</i> + +	21	—	—	—	14.5
+ + <i>leu ser</i>	—	—	—	8	
<i>arg</i> + + <i>ser</i>	—	10	—	—	5.5
+ <i>phe leu</i> +	—	—	1	—	
Total no. of recombinants per plate	72	197	10	180	
No. of recombinants examined	207	228	213	232	

percentage frequencies of the seven pairs of genotypes (or the crossover patterns creating them) in the three crosses were compared for the two possible arrangements of the four markers (Table 5). The data clearly favour the Fig. 1(a) arrangement, which results in a significantly lower frequency of multiple crossover genotypes (an average frequency of quadruple crossovers of 1% for the Fig. 1(a) arrangement compared with 4.2% for the Fig. 1(b) arrangement).

The total relative recombination frequencies in each of the four intervals between adjacent markers, and the average for the three crosses (lower part of Table 5) again clearly shows linkage between *arg-8* and *ser-1*. A linkage between *leu-1* and *ser-1* is also indicated. These calculations also fit with Fig. 1a.

Table 5. Summary of crosses in Tables 2, 3 and 4

Values in parentheses are percentages. *a* Refers to the arrangement of loci in Fig. 1(a) and *b* to the arrangement in Fig. 1(b).

Crossovers in intervals (see Fig. 1)	Mean frequencies of pairs of complementary genotypes						Mean value of <i>a</i> (%)
	E1 × R24 (Table 2)		R74 × R75 (Table 3)		R73 × R76 (Table 4)		
	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	
1, 2	10.5 (5.1)	10.5 (5.1)	1 (1)	1 (1)	1.5 (0.7)	1.5 (0.7)	(2)
1, 3	86.5 (42.4)	86.5 (42.4)	8 (8)	8 (8)	14.5 (6.5)	14.5 (6.5)	(19)
1, 4	4.5 (2.2)	74.5 (36.5)	8 (8)	72 (72)	37.5 (17)	155.5 (69)	(9)
2, 3	22 (10.8)	22 (10.8)	4 (4)	4 (4)	4.5 (2)	4.5 (2)	(6)
2, 4	4.5 (2.2)	1.5 (0.7)	8 (8)	0 (0)	5.5 (2.4)	5 (2.2)	(4)
3, 4	74.5 (36.5)	4.5 (2.2)	72 (72)	8 (8)	155.5 (69)	37.5 (17)	(59)
1, 2, 3, 4	1.5 (0.7)	4.5 (2.2)	0 (0)	8 (8)	5 (2.2)	5.5 (2.4)	(1)
Totals	204 (100)	204 (100)	101 (100)	101 (100)	224 (100)	224 (100)	(100)

Total frequencies of crossovers in each interval (arrangement *a*)

Interval	E1 × R24 (Table 2)	R74 × R75 (Table 3)	R73 × R76 (Table 4)	Mean
1 (<i>ser-1-leu-1</i>)	50	17	26	31 (20%)
2 (<i>arg-8-ser-1</i>)	19	13	7.5	13 (8%)
3 (<i>arg-8-phe-1</i>)	90	84	80	85
4 (<i>leu-1-phe-1</i>)	42	88	91	74 } 79.5 (50%)

Fig. 1. Two possibilities for the arrangement of four markers from the relative recombination frequencies in the six intervals from Tables 2, 3 and 4.

Multi-factor crosses

After evidence was obtained for a circular arrangement of the four markers, further crosses between various strains and involving additional markers were done. Nearly all of these multiply marked strains were recombinants that had been constructed by matings or by protoplast fusions between various double and triple auxotrophic mutants. Typical multi-factor crosses involved five to seven markers. Spores from mixed cultures of the two strains to be crossed were usually seeded on three or four different media to select for recombination in different map intervals.

After analysis of the recombinants recovered from the selective media, the ratio of each non-selected pair of marker alleles within the arc between the selected markers was determined following the rationale outlined by Hopwood (1967). Of the two possible positions, the one chosen needed the minimum numbers of multiple crossovers for the production of the observed recombinant genotypes. In other cases the most probable position was deduced from consideration of the segregation of the respective allele pair with that of known adjacent markers. Back-crosses involving the same or other known markers in different couplings were performed; these confirmed the correct location of previously mapped mutations. Table 6 shows

Table 6. Analysis of a multi-factor cross of *S. chrysomallus* strains 2082 (*his-1 pro-1 ura-1*) and R25 (*arg-2 leu-1 phe-2*)

In the diagrams the numbers next to the markers are frequencies of alleles, the numbers between the circles designate map intervals, and the triangles indicate selected alleles.

Genotypes	No.	Crossover in intervals
Selection: <i>his-1</i> ⁺ / <i>leu-1</i> ⁺		
+ + + +	22	2, 5
<i>arg</i> + + +	48	3, 5
+ <i>phe</i> + +	6	2, 3, 4, 5
+ + <i>pro</i> +	2	2, 6
+ + + <i>ura</i>	21	1, 5
<i>arg phe</i> + +	16	4, 5
<i>arg</i> + <i>pro</i> +	37	3, 6
+ <i>phe</i> + <i>ura</i>	8	1, 3, 4, 5
<i>arg phe pro</i> +	34	4, 6
Total	194	
Selection: <i>phe-1</i> ⁺ / <i>ura-1</i> ⁺		
+ + + +	7	2, 5
<i>arg</i> + + +	13	3, 5
<i>arg leu</i> + +	127	3, 4
<i>arg</i> + + <i>pro</i>	31	3, 6
+ <i>leu his</i> +	1	1, 2, 4, 6
<i>arg leu</i> + <i>pro</i>	2	3, 4, 5, 6
Total	181	
Selection: <i>arg-2</i> ⁺ / <i>pro-1</i> ⁺		
+ + + +	2	2, 5
+ <i>leu</i> + +	3	2, 4
+ + + <i>ura</i>	6	1, 5
<i>phe leu</i> + +	21	2, 3
+ <i>leu</i> + <i>ura</i>	8	1, 4
<i>phe leu</i> + <i>ura</i>	109	1, 3
+ <i>leu his ura</i>	1	4, 6
<i>phe</i> + <i>his ura</i>	1	3, 4, 5, 6
<i>phe leu his ura</i>	6	3, 6
Total	161	

the results of a typical six-factor cross between *S. chrysomallus* strains 2082 (*his-1 ura-1 pro-1*) and R25 (*leu-1 arg-2 phe-1*). The analysis of the recombinants from each of the three selective media allowed calculation of the allele ratios of the four non-selected markers. The sequence of markers was the same in all three cases. The most probable arrangement of markers was determined from the map order that gave the lowest number of multiple crossovers (not shown). In all three cases the sequence was the same.

Table 7. Results of a mating and of a protoplast fusion between *S. chrysomallus* strains R26 (*arg-2 his-1 leu-1*) and R36 (*ade-1 phe-1 pdx-1*)

In the diagrams the numbers next to the markers are frequencies of alleles, the numbers between the circles designate map intervals, and the triangles indicate selected alleles.

Selection: *ade-1*⁺/*his-1*⁺

Genotypes				Mating	Protoplast fusion	Crossover in intervals
+	+	+	+	10	25	2, 5
<i>leu</i>	+	+	+	1	4	2, 6
+	<i>arg</i>	+	+	11	27	1, 5
+	+	+	<i>pdx</i>	105	66	3, 5
<i>leu arg</i>	+	+		2	8	1, 6
<i>leu</i>	+	+	<i>pdx</i>	17	12	3, 6
<i>leu</i>	+	<i>phe pdx</i>		0	2	3, 4, 5, 6
+	+	<i>phe pdx</i>		4	2	3, 4
+	<i>arg</i>	+	<i>pdx</i>	0	2	1, 2, 3, 5
+	<i>arg phe</i>	+		0	2	1, 4
Total				150	150	

Mating				Protoplast fusion			
<i>arg</i> ⁺	<i>leu</i> ⁺	<i>leu</i>	<i>pdx</i> ⁺	<i>arg</i> ⁺	<i>leu</i> ⁺	<i>leu</i>	<i>pdx</i> ⁺
	119	18	11		93	18	29
<i>arg</i>	11	2	13	<i>arg</i>	31	8	37
	not adjacent		adjacent		not adjacent		adjacent

Mating

Protoplast fusion

Analysis of crosses by protoplast fusion

Protoplast fusion has been shown to be valuable for mapping studies in several cases (Hopwood & Wright, 1978; Baltz, 1980; Hranueli *et al.*, 1983; Hopwood *et al.*, 1983). During protoplast fusion an increase in the frequency of multiple crossover genotypes occurs compared to those in corresponding matings. In view of the low sporulation of *S. chrysomallus* and the high recombination frequencies observed during protoplast fusions with this strain (Keller *et al.*, 1983), we tried to apply this technique for mapping studies in multi-factor crosses. Table 7 illustrates the results of crosses based on mating and protoplast fusion between *S. chrysomallus* strains R26 (*leu-1 arg-2 his-1*) and R36 (*phe-1 ade-1 pdx-1*). Protoplast fusion clearly yielded more different genotypes than obtained in a mating, and these belonged to the rare classes, as expected. There was also a significant increase in frequency of crossing over in the different intervals (particularly between *his-1 arg-2*) which leads to the allele ratios being closer to 50:50 in the protoplast fusion than in the mating. Similar effects were described by Hopwood *et al.* (1983) in protoplast fusions with *Streptomyces lividans*. The data allow the position of an unknown marker in this cross (say *arg-2*) to be mapped by consideration of its co-segregation

Fig. 2. Arrangement of various marker loci on the genetic map of *S. chrysomallus*. The *ilv-1* marker has only been mapped between the *phe-1* and *leu-1* loci.

with other loci. The 2×2 tables for both mating and protoplast fusion clearly indicate a location of the *arg-2* mutation near to *pdx-1*. Thus, protoplast fusion appears to be a useful technique for mapping studies. However, several protoplast fusion crosses yielded results contradictory to those obtained in conventional matings. In such crosses certain alleles (not always the same ones) appeared to be over-represented, leading to a false allele ratio and hence to a different position on the genetic map. We found earlier (Keller *et al.*, 1983) that actinomycin production has some negative influence on the regeneration of antibiotic non- or low-producers when fused with actinomycin producers. Apparently this effect played a role in such protoplast fusions, because the effects described were always observed when there was strong actinomycin synthesis in the regeneration plates (results not shown). That such effects were not observed in matings may be due to the fact that actinomycin production was very low on MM but was high on modified R2 medium, especially when yeast extract was present. These findings led to problems in using the results of protoplast fusions for mapping purposes with mutants showing increased actinomycin synthesis on modified R2. Therefore, protoplast fusions were mostly performed for strain constructions (up to 70% recombinants in non-selective conditions), or in rare cases (with actinomycin low-producers) for confirmation of data obtained by matings.

The genetic map, linkage and fertility of S. chrysomallus

As a result of about 70 crosses between various mutants of *S. chrysomallus*, a linkage map could be constructed (Fig. 2). Although the relative map distances between most markers could not be determined precisely (because most crosses were multi-factor crosses analysed by the allele-ratio method), we obtained evidence for the arrangement presented by several semi-quantitative observations made during various multi-factor crosses. From the numbers of colonies on selective media and the segregation of pairs of non-selected alleles with those of the four markers in Fig. 1, it was possible to show linkage with the four known markers. Furthermore, some parental marker pairs almost always segregated together (e.g. *phe-1* and *ade-1* or *cys-1* and *leu-1*). It was therefore assumed that they were closely linked. Moreover, in a variety of crosses, selection for short intervals resulted in an increase in the proportion of heterokaryons (often also detectable by their smaller colony size). (This effect made it possible to perform complementation tests between independently isolated mutations with identical requirements, as described by Hopwood (1967), in order to see whether they were allelic or not.) Thus, the appearance of heterokaryons at a higher frequency indicated that the two loci for which selection was made were closer to each other than other loci for which the selection gave no heterokaryons or low frequencies of heterokaryons.

Our data revealed only a few markers closely linked to *phe-1*. The majority of mutations mapped in the region between *leu-1* and *arg-1,2,8*. In all relevant crosses performed, the interval between *phe-1* and the *pab-1-cys-1-leu-1* region was large. However, the intervals *his-1-phe-1* and *arg-1,2,8-phe-1* appeared to be significantly larger. The markers *ade-1*, *met-4* and *thr-3* mapped rather closely to the *phe-1* locus and it was therefore concluded that these four markers lay in a region of the chromosome significantly distant from that of the majority of mutations.

DISCUSSION

The results presented here reveal a circular linkage map for *Streptomyces chrysomallus*. Similar linkage groups have been described previously for *S. coelicolor* A3(2) (Hopwood, 1965) and a series of other streptomycetes (Friend & Hopwood, 1971; Coats & Roeser, 1971; Alačević *et al.*, 1973; Baumann *et al.*, 1974; Hopwood *et al.*, 1983). The analysis of haploid recombinants recovered from crosses by matings, and to a limited extent from protoplast fusions, proved to be

useful for mapping. The appearance of heterokaryons did not disturb the analysis because their numbers were usually low. Although relative map distances between four markers could be determined in a trio of four-factor crosses according to the procedure of Baumann *et al.* (1974), the linkage relations of all other markers were only roughly analysed, by consideration of their positions relative to other loci, and from their segregational behaviour in respect of the four known markers. The appearance of heterokaryons on certain selective media was in most cases a good indicator of closely linked selected markers, and so a linkage map could be constructed (Fig. 2). On comparing this genetic map with that of *S. coelicolor*, the genetically best investigated streptomycete (Hopwood *et al.*, 1973), and assuming the identity in the two organisms of the loci considered, a strong conservation of gene sequences is evident (Fig. 3).

The low fertility in *S. chrysomallus* did not severely hamper its genetic analysis. The apparent fertility (10^{-9} – 5×10^{-6}) was highly variable, but was generally lower than that reported for IF $\times$ IF (SCP1⁺ $\times$ SCP1⁺) crosses in *S. coelicolor* (Vivian & Hopwood, 1970), or SRP1⁺ $\times$ SRP1⁺ crosses in *Streptomyces rimosus* (Friend & Hopwood, 1971; Friend *et al.*, 1978). Covalently closed circular DNA could not be detected by physical detection methods in *S. chrysomallus*, and no evidence was obtained that the particular strains used in this study showed an enhanced fertility, i.e. the fertility of a strain varied from cross to cross. Furthermore, *S. chrysomallus* did not display a significantly increased chromosomal transfer when harbouring the plasmid pIJ303, indicating a difference from the streptomycetes of the *S. coelicolor*/*S. lividans* group. Neither plasmid pIJ303 nor pIJ211 (Kieser *et al.*, 1982) induced pock formation even in well-sporulating strains of *S. chrysomallus* (not shown), and it is possible that this lack of expression of the Ltz⁺ phenotype was due to the presence of a still unknown native plasmid in the organism. However, the finding that these plasmids replicated and expressed their antibiotic resistance in *S. chrysomallus* makes this strain suitable for gene cloning. Thus, the data presented provide a good background of genetics for future work on actinomycin synthesis.

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