Genetic Analysis in *Streptomyces ambofaciens*

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A chromosomal linkage map of ten markers was established for *Streptomyces ambofaciens* by the four-factor cross method and allele-gradient analysis. Mutants were obtained by nitrous acid treatment as well as by UV mutagenesis. The fertility of crosses was enhanced over 100-fold by pSAM2, a plasmid present in some strains of *S. ambofaciens*, and over 1000-fold by the conjugative plasmid pIJ303.

INTRODUCTION

Streptomyces ambofaciens is used for the industrial production of the macrolide antibiotic spiramycin, and the development of its genetics has several applications: a study of the relationships between auxotrophic mutants and their biochemical defects, which have seldom been described in other *Streptomyces* spp., the mapping of antibiotic-production genes, and a study of the biology of the plasmids of this species. In our laboratory, a plasmid pSAM2 (11 kb) of *S. ambofaciens* has been described (Pernodet *et al.*, 1984). In different strains of *S. ambofaciens* pSAM2 can exist either solely in an integrated form or in both free and integrated forms. A strain devoid of any plasmid sequence was identified and the transformation of this strain with pSAM2 has been demonstrated. The transfer of pSAM2 was detected by the lethal zygosis reaction or 'pock' formation (Hopwood & Wright, 1973; Bibb *et al.*, 1977; Kieser *et al.*, 1982). The integration of this plasmid occurred through specific plasmid and chromosome sites (Simonet *et al.*, 1987). To further investigate the biology of this plasmid we wished to map the chromosomal integration site and establish whether the plasmid acted as a fertility factor. For these purposes it was first necessary to establish a linkage map for the strain. There are numerous species of actinomycetes and only a few linkage maps have been established (for a recent review, see Rhodes, 1986), mostly for species for which there was an interest in mapping antibiotic-biosynthesis genes. In order to establish a genetic map, many mutants have to be isolated, from which a collection of double or triple mutants must be constructed.

This paper describes some instability properties of auxotrophic mutants obtained by UV mutagenesis, the use of nitrous acid as a mutagen (not previously described in actinomycetes; Baltz, 1986), the promotion of fertility by plasmid pSAM2 and by another plasmid pIJ303, and the construction of the first genetic map for *S. ambofaciens*.

METHODS

Strains. All our mutants were derived from *Streptomyces ambofaciens* DSM 40697 devoid of plasmid pSAM2. These strains and their derivation are listed in Table 1.

Plasmids. pIJ303, derived from pIJ101 (Kieser *et al.*, 1982), a *S. lividans* plasmid, was kindly provided by D. A. Hopwood. pSAM2 was isolated from *S. ambofaciens* JI3212 (Pernodet *et al.*, 1984).

Media. Complete medium (CM) for maintenance of strains, sporulation and mutant isolation was Hickey Tresner medium (Pridham *et al.*, 1957). Minimal medium (MM) was a slight modification of a medium used for filamentous fungi (Esser, 1974). It contained, per litre, KH₂PO₄ 0.25 g, K₂HPO₄ 0.34 g, MgCl₂ · 6H₂O 0.25 g, urea 0.5 g, fructose 2% (w/v), and 0.05 ml of a solution of trace elements containing, per litre, CuSO₄ · 5H₂O 5 g,

Table 1. *List of strains derived from DSM 40697*

Strain	Genotype	Origin
OS105	<i>ilv-1</i>	Ethylenimine mutagenesis
OS106	<i>ilv-1 spc-1</i>	Spontaneous Sp^{R} mutant of OS105
OS110	<i>his-1</i>	UV mutagenesis
OS111	<i>met-1</i>	UV mutagenesis
OS500	<i>ura-1</i>	Spontaneous 5-fluoroorotate-resistant mutant
OS501	<i>leu-1</i>	HNO_2 mutagenesis
OS502	<i>leu-1</i> pIJ303	Transformation of OS501
OS503	<i>ade-1</i>	HNO_2 mutagenesis
OS504	<i>ade-1</i> pIJ303	Transformation of OS503
OS505	<i>nic-1</i>	HNO_2 mutagenesis
OS506	<i>nic-1</i> pIJ303	Transformation of OS505
OS507	<i>nic-2</i>	HNO_2 mutagenesis
OS508	<i>lys-1</i>	HNO_2 mutagenesis
OS112	<i>ura-1 ilv-1 spc-1</i>	Protoplast fusion of OS106 and OS500
OS113	<i>met-1 ilv-1 spc-1</i>	Protoplast fusion of OS106 and OS111
OS114	<i>leu-1 his-1</i>	Protoplast fusion of OS501 and OS110
OS115	<i>nic-1 ilv-1 spc-1</i>	Protoplast fusion of OS106 and OS505
OS509	<i>leu-1 his-1 met-1 ilv-1 spc-1</i>	Protoplast fusion of OS114 and OS113
OS510	<i>leu-1 his-1 met-1 ilv-1 spc-1</i> pIJ303	Plasmid transfer from cross AO
OS116	<i>nic-1</i> pSAM2	Transformation of OS505
OS117	<i>leu-1</i> pSAM2	Transformation of OS501

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 10 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 200 g, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 20 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 10 g, NaCl 6 mg. Fructose was autoclaved separately as a 50% (w/v) solution and added to the medium before pouring the plates. Nutritional requirements were added at a concentration of 50 mg l^{-1} , spectinomycin at 50 mg l^{-1} in MM and 100 mg l^{-1} in CM, 5-fluoroorotate at 2 mg ml^{-1} and nosiheptide (a generous gift from Rhône-Poulenc Santé) at 30 mg l^{-1} .

Protoplasts and transformation. Protoplasts were prepared from mycelium grown in YEME (Bibb *et al.*, 1977) and transformation of protoplasts was performed according to Bibb *et al.* (1978). R2YE medium (Thompson *et al.*, 1980) was used for protoplast regeneration and for the detection of 'pocks'. Protoplast fusion was performed as described by Hranueli *et al.* (1983).

Culture conditions. In order to shorten the time of each experiment, plates were incubated at 37°C (growth is more rapid than at 28°C and sporulation is unaffected), except for protoplast regeneration which was performed at 28°C .

Crosses. Equal mixtures of spores of the two parent strains were mixed on a CM plate and incubated for 3–5 d. The spores were then collected and filtered through a cotton plug in a disposable Pasteur pipette.

Mutagenesis. For UV treatment, a diluted suspension of spores (10 ml ; $10^6 \text{ spores ml}^{-1}$) was irradiated with a germicidal UV lamp, with constant agitation. A UV flux of $100\text{--}300 \text{ J m}^{-2}$ was used.

For ethylenimine treatment, 10 ml of a suspension of $5 \times 10^6 \text{ spores ml}^{-1}$ was treated with 0.4 ml ethylenimine (Serva) for 25 min (Maljina & Wolf, 1980).

For nitrous acid treatment, 1 ml of a suspension of $2\text{--}5 \times 10^8 \text{ spores ml}^{-1}$ was centrifuged and washed twice with sterile water. The pellet was resuspended in 5 ml 0.1 M -citrate buffer (pH 3.0), kept in an ice bath for 5 min and then 0.05 ml 2 M - NaNO_2 was added. The spores were incubated at 0°C . At different intervals, up to 120 min, samples were removed and neutralized by addition of an equal volume of 0.2 M - K_2HPO_4 and serial dilutions were performed to obtain a viable count. After 3 d, grown colonies were replica-plated on MM and CM to score for presumptive auxotrophs.

Southern hybridization. This was as described before (Pernodet *et al.*, 1984).

RESULTS

Isolation of mutants

When UV mutagenesis was used, there was a correlation between the decrease of viability and the increase of presumptive auxotrophs, as might be expected. At a UV flux of 250 J m^{-2} the surviving fraction was 1.3% and we obtained 1.3% of presumptive auxotrophs. Each one was subsequently streaked on CM and then replica-plated again on the same two media. After this step, about half appeared to be auxotrophs, some proved to be prototrophs and were discarded, while others segregated prototroph and auxotroph colonies. These last auxotroph segregants were further analysed. In many cases some of these presumptive auxotrophs continued to

Table 2. *Effect of pSAM2 and pIJ303 on chromosomal recombination*

Cross		Frequency of prototrophs*
AJ1 OS505 Nic ⁻ (p ⁻) × OS501 Leu ⁻ (p ⁻)		1.5 × 10 ⁻⁷
AK2 OS116 Nic ⁻ (p ⁺) × OS117 Leu ⁻ (p ⁺)	} p = pSAM2	3.0 × 10 ⁻⁵
AK3 OS116 Nic ⁻ (p ⁺) × OS501 Leu ⁻ (p ⁻)		3.4 × 10 ⁻⁵
AK4 OS505 Nic ⁻ (p ⁻) × OS117 Leu ⁻ (p ⁺)		5.6 × 10 ⁻⁵
AJ2 OS506 Nic ⁻ (p ⁺) × OS502 Leu ⁻ (p ⁺)		2.5 × 10 ⁻⁴
AJ3 OS506 Nic ⁻ (p ⁺) × OS501 Leu ⁻ (p ⁻)	} p = pIJ303	8.3 × 10 ⁻⁴
AJ4 OS505 Nic ⁻ (p ⁻) × OS502 Leu ⁻ (p ⁺)		7.3 × 10 ⁻⁴

* Calculated as the number of colonies on MM compared to the number of parental colonies on CM.

segregate auxotroph and prototroph colonies when re-streaked a second or third time. It is difficult to analyse this apparent instability at the present stage of our work.

For chemical mutagenesis, except for one mutant, we avoided the use of mutagens which are carcinogenic, and chose nitrous acid, which is comparatively safe. Mutagenesis was efficient and results were very reproducible: 1% of auxotrophs were obtained with treatments giving approximatively 0.4% viability, and these auxotrophs were much more stable than those obtained after UV mutagenesis. A single re-streaking step was sufficient to obtain stable auxotrophs.

Other classes of mutants were obtained by positive selection. Spontaneous mutants were isolated resistant to spectinomycin, or to 5-fluoroorotate, an antimetabolite.

Fertility factors

Most *Streptomyces* plasmids studied so far behave as conjugative sex plasmids (Hopwood *et al.*, 1984). Different strains of *S. ambofaciens*, belonging to two lineages, have been studied in our laboratory (Pernodet *et al.*, 1984). One of them, ATCC 23877, harbours two plasmids, pSAM1 (80 kb) and pSAM2 (11 kb); the other strain, DSM 40697, from which all our mutants were derived, harbours neither of these plasmids. To find out whether pSAM2 behaved as a conjugative sex plasmid, we introduced it by transformation into several auxotrophic derivatives of DSM 40697. Transformants were detected by the lethal zygosis reaction or 'pock' formation. The presence of pSAM2 in the transformed strain was confirmed by Southern hybridization with a ³²P-labelled pSAM2 probe. Pairs of complementary auxotrophs in which one or both parents carried pSAM2 were crossed and the frequency of prototrophic recombinants was scored.

As shown in Table 2, pSAM2 behaved as a conjugative sex plasmid when present in one or both parents in the cross. The same kind of experiments were done to find out whether pIJ303, a derivative of the plasmid pIJ101 from *S. lividans*, behaves as a conjugative plasmid in *S. ambofaciens*, as it does in some *Streptomyces* species (Kieser *et al.*, 1982) but not in *S. chrysomallus* (Keller *et al.*, 1985). In this case transformants were detected by resistance to nosiheptide, as the *tsr* gene carried by pIJ303 confers resistance to both thiostrepton and nosiheptide (Cundliffe & Thompson, 1981). Table 2 shows that pIJ303 did behave as a conjugative sex plasmid in *S. ambofaciens*, when present in one or both parents.

Four-factor crosses

The principles of genetic mapping in *Streptomyces* have been established by Hopwood (1972). The prerequisite is a pair of parental strains differing in four selectable markers, two in each parent. There are two ways to obtain the parental multiply-marked strains, either successive steps of mutagenesis or recombination. The disadvantage of the first method is that two steps of mutagenesis may decrease the viability and sporulation of the strain. Constructing multiply-marked strains by recombination is preferable in the sense that one can follow the same allele throughout all crosses. The development of protoplast fusion in *Streptomyces* (Hopwood *et al.*,

Table 3. Analysis of the four-factor cross *ilv-1 met-1 spc-1* × *leu-1* (pIJ303)

Genotype of selectable progeny	Frequencies* of genotypes on selective media supplemented with:								Mean frequency of each pair of complementary genotypes†
	Methionine		Isoleucine + valine		Methionine, leucine and spectinomycin		Isoleucine + valine, leucine and spectinomycin		
	A	B	A	B	A	B	A	B	
+ + + <i>spc</i>	9	252	9	186	121	290	81	238	241
+ + + +	138	3864	140	2893	—	—	—	—	3378
<i>ilv</i> + + + <i>spc</i>	—	—	1	21	—	—	45	132	76
+ <i>met</i> + + <i>spc</i>	0	0	—	—	6	14	—	—	7
+ + <i>leu</i> <i>spc</i>	—	—	—	—	23	55	14	41	48
<i>ilv</i> + + +	—	—	0	0	—	—	—	—	0
+ <i>met</i> <i>leu</i> <i>spc</i>	—	—	—	—	0	0	—	—	
+ <i>met</i> + +	3	84	—	—	—	—	—	—	57
<i>ilv</i> + <i>leu</i> <i>spc</i>	—	—	—	—	—	—	10	29	
Sample size	150		150		150		150		
Total recombinants per unit volume		4200		3100		359		440	

Segregation of nonselected allele pairs‡									
	<i>met</i> ⁺	<i>met</i>		<i>ilv</i> ⁺	<i>ilv</i>		<i>met</i> ⁺	<i>met</i>	
<i>spc</i> ⁺	138	3	<i>spc</i> ⁺	140	0	<i>leu</i> ⁺	121	6	<i>leu</i> ⁺
<i>spc</i>	9	0	<i>spc</i>	9	1	<i>leu</i>	23	0	<i>leu</i>
χ^2 :	0.2			14.1			1.1		
									0.3

Relative locations:	Non-adjacent	Adjacent		Adjacent	Non-adjacent
	Relative recombination frequencies in each interval§				
<i>ilv</i> – <i>met</i>	<i>ilv</i> – <i>spc</i>	<i>ilv</i> – <i>leu</i>	<i>met</i> – <i>spc</i>	<i>met</i> – <i>leu</i>	<i>spc</i> – <i>leu</i>
140	296	3683	422	3695	3483

* Columns A, number of recombinants per sample; columns B, number of recombinants per unit volume.

† From columns B.

‡ From columns A in main table.

§ Calculated from the mean frequency of each pair of complementary genotypes: last column in main table.

1977) allows the isolation of recombinant strains at frequencies greater than 1% of regenerating protoplasts.

Starting with an *ilv-1 spc-1* strain and several different single auxotrophs, protoplast fusion was used to obtain several doubly- and triply-marked strains of *S. ambifaciens*, e.g. *met-1 ilv-1 spc-1*, and *ura-1 ilv-1 spc-1* strains. For the mapping experiments, done by conventional strain crosses (Hopwood *et al.*, 1985), pIJ303 was used to increase recombination frequency since its presence could be detected by its resistance to nosisheptide (*tsr* gene). Since plasmid transfer in protoplast fusion or crosses is very efficient, most recombinants obtained carried pIJ303.

An example of a four-factor cross is summarized in Table 3. The next step was the choice of the best arrangement of markers between the three possibilities shown in Fig. 1. In each medium (Table 4) the segregation of non-selected allele pairs was tabulated in correlation with the number of crossovers required for each alternative arrangement. The arrangement which requires the minimum number of cross-overs represents the most likely gene order. Arrangement C in Fig. 1 is the one which best fits this prediction. Many other crosses were analysed in the same way (results not shown) and the relative position of different markers could be unambiguously established. Representative crosses and their significance in ordering loci are listed in Table 5.

Fig. 1. Alternative arrangements of markers in the cross *ilv-1 met-1 spc-1* × *leu-1*.

Table 4. Correspondence between the segregation of an allele-pair and the number of crossovers required for the three possible arrangements shown in Fig. 1

Medium supplemented with	Segregation of non-selected allele pairs	Number of crossovers required for each alternative arrangement of Fig. 1		
		A	B	C
Methionine	<i>Spc^S Met⁺</i> 138	2	2	2
	<i>Spc^S Met⁻</i> 3	4	2	2
	<i>Spc^R Met⁺</i> 9	2	4	2
	<i>Spc^R Met⁻</i> 0	2	2	2
Isoleucine + valine	<i>Spc^S Ilv⁺</i> 140	2	2	2
	<i>Spc^S Ilv⁻</i> 0	2	2	4
	<i>Spc^R Ilv⁺</i> 9	2	4	2
	<i>Spc^R Ilv⁻</i> 1	2	2	2
Leucine, methionine and spectinomycin	<i>Leu⁺ Met⁺</i> 121	2	4	2
	<i>Leu⁺ Met⁻</i> 6	2	2	2
	<i>Leu⁻ Met⁺</i> 23	2	2	2
	<i>Leu⁻ Met⁻</i> 0	2	2	4
Leucine, isoleucine + valine and spectinomycin	<i>Leu⁺ Ilv⁺</i> 81	2	4	2
	<i>Leu⁺ Ilv⁻</i> 45	2	2	2
	<i>Leu⁻ Ilv⁺</i> 14	2	2	2
	<i>Leu⁻ Ilv⁻</i> 10	4	2	2

Table 5. Mapping crosses performed and their significance in ordering loci

Cross	Order of loci inferred	Genes closely linked
FA OS113 × OS502	<i>spc-1 leu-1 met-1 ilv-1</i>	None
AC OS112 × OS502	<i>spc-1 leu-1 ilv-1 ura-1</i>	<i>ura-1</i> and <i>ilv-1</i>
AD OS112 × OS506	<i>spc-1 leu-1 ilv-1 nic-1</i>	<i>nic-1</i> and <i>ilv-1</i>
AG OS115 × OS504	<i>spc-1 ade-1 ilv-1 nic-1</i>	<i>nic-1</i> and <i>ilv-1</i>
AH OS115 × OS502	<i>spc-1 leu-1 ilv-1 nic-1</i>	<i>nic-1</i> and <i>ilv-1</i>
AO OS509 × OS504	<i>spc-1 leu-1 his-1 met-1 ade-1 ilv-1</i>	None
FB OS510 × OS507	<i>spc-1 leu-1 nic-2 his-1 met-1 ilv-1</i>	None
FC OS510 × OS508	<i>spc-1 lys-1 leu-1 his-1 met-1 ilv-1</i>	None

Mapping by single selection

A multiply-marked strain carrying *ilv-1 met-1 his-1 leu-1 spc-1*, obtained from a protoplast fusion between *ilv-1 met-1 spc-1* and *his-1 leu-1* strains, was crossed with another strain carrying *ade-1* (the same allele already used in many four-factor crosses). Two single selections were

Fig. 2. Allele-gradient analysis in a cross *ilv-1 met-1 his-1 leu-1 spc-1* $\times$ *ade-1*. Triangles denote markers selected. (a) Selection for Spc^R His^+ ; (b) selection for Spc^R Leu^+ .

Fig. 3. Relative gene location in the linkage map of *S. ambofaciens*. Markers are spaced on the figure in accordance with results from allele-gradient analysis, but intervals represented on the map are not intended to be proportional. Nevertheless, *ura-1*, *nic-1* and *ilv-1* are closely linked as judged by the results from four-on-four crosses.

studied and the results were subjected to allele-gradient analysis (Hopwood *et al.*, 1985). These results (Fig. 2) confirmed the previous relative location of the markers.

Chromosomal linkage map

The linkage map of *S. ambofaciens* deduced from ten four-factor crosses and from allele-gradient analysis is shown in Fig. 3. From the relative recombination frequency in each interval it is possible to estimate the distances between some genes, especially for linked markers like *ura-1*, *nic-1* and *ilv-1*.

DISCUSSION

These studies led to the construction of a simple genetic map of *S. ambofaciens*, involving ten markers. The map could easily be extended to cover new markers.

We showed that UV mutagenesis gives rise, in some cases, to highly unstable auxotrophs that revert to prototrophy at high frequency. This might not be a specific feature of *S. ambofaciens*, but a more general situation encountered in other *Streptomyces* spp. Nevertheless we obtained a few stable auxotrophs. In order to avoid the use of carcinogenic chemical mutagens, we isolated

auxotrophic mutants by nitrous acid treatment. The mutants so obtained were much more stable than those obtained by UV mutagenesis. We also selected spontaneous mutants resistant to 5-fluoroorotate, which has been used in yeast (Boeke *et al.*, 1984) to isolate uracil-requiring mutants. In addition, a spontaneous mutant of the *ilv* auxotroph resistant to the antibiotic spectinomycin was isolated. This doubly-marked strain was then used to generate triple mutants by protoplast fusion with other auxotrophs.

Protoplast fusion is very useful for generating doubly- or multiply-marked strains, but conventional crosses are preferred for mapping, since protoplast fusion gives a relatively high proportion of multiple crossovers and therefore complicates interpretation of data. The four-factor cross procedure requires a pair of parental strains differing in four selectable markers. There are two possibilities: either the parental strains are doubly auxotrophic, or one is a triple mutant bearing two auxotrophies and one resistance marker, and the other parent can be a single auxotrophic mutant. In the first case, in order to obtain the required parental strains, two protoplast fusion steps are needed, one for each parent. In the second case a single protoplast fusion step is sufficient for the construction of the triple mutant. Moreover, when one triple mutant is obtained, it can be crossed with many single mutants and a first preliminary map can be easily constructed.

The natural frequency of recombination by conjugation is quite low in many *Streptomyces* spp. (10^{-7} in *S. ambofaciens*). We therefore investigated the influence of plasmids on fertility. We showed that pSAM2, a plasmid present either solely in the integrated form or as free and integrated plasmid in some strains of *S. ambofaciens*, could increase the frequency of recombination 100-fold when introduced by transformation into one or both parents of a cross (Table 2), like SCP2*, pIJ6, pIJ303, pIJ110, pIJ408, pIJ928 or pWOR191 in *S. lividans* (Hopwood *et al.*, 1984). The plasmid pIJ303, a thiostrepton-resistance-determining derivative of pIJ101 also acted as a fertility factor in *S. ambofaciens*. This plasmid is known to act as a fertility factor in many *Streptomyces* spp. Our mapping crosses were done using this last plasmid, as its presence could be detected by the resistance of the strain to nosiheptide. The same procedure described above for the four-factor crosses can be applied to multifactor crosses, with the advantage that allele-gradient analysis can be done. The only limiting step is the construction of a multiply-marked strain which is obtained through two or more steps of protoplast fusion or crossing. In a cross of a multiply-marked strain with a single mutant, gradient analysis can be done using selection by two markers, one of each parent strain; for any one cross, many double selections can be carried out (two are shown in Fig. 2). In each selection medium all the recombinants can be classified in term of non-selected markers by the replica-plating technique. Then the data can be analysed in two ways: either in a classical allele-gradient or as a series of multiple four-factor crosses. In the case illustrated (Fig. 2), when the selected markers are His⁺ and Spc^R, it is possible to analyse the data as if they were derived from the crosses *leu-1 his-1 spc-1* × *ade-1*, or *met-1 his-1 spc-1* × *ade-1*, or *ilv-1 his-1 spc-1* × *ade-1*. Using the same type of analysis as shown in Table 3 and Fig. 1, the different alleles can be ordered more reliably.

It is premature to compare the circular chromosomal map of *S. ambofaciens* shown in Fig. 3 to maps described in other *Streptomyces* species, since very little is known about the relationship between the different auxotrophic mutants and their biochemical defects. Thus it is not possible to reliably compare the limited number of markers used in this study with known loci of *S. coelicolor* A3(2), the best-characterized *Streptomyces* strain (Hopwood *et al.*, 1985).

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