A Plasmid Involved in Chloramphenicol Production in *Streptomyces venezuelae*: Evidence from Genetic Mapping

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SUMMARY

To test the hypothesis that chloramphenicol production in *Streptomyces venezuelae* depends on the presence of a plasmid, mapping analysis was carried out by using eight markers in addition to chloramphenicol production and melanoid pigment formation. The sequence of the eight markers was determined on a circular linkage map as follows: *-his-ade-str-leu-lys-met-ilv-pro-(his-)*. This sequence resulted in the frequency of quadruple crossover (q.c.o.) recombinants having the lowest value, 3.2 to 4.9 %. However, the character of chloramphenicol non-production, which was obtained by incubating mycelia with acriflavin, was not located on this linkage map; more than 15 % q.c.o. recombinants would have been required to explain the results. From these results and other tests, it is concluded that chloramphenicol production is controlled by a plasmid. This plasmid appeared to be non-transferable in conjugation.

INTRODUCTION

It has been reported that tyrosinase in *Streptomyces scabies* is coded by a plasmid (Gregory & Shyu, 1961; Gregory & Huang, 1964) and that fertility in *Streptomyces coelicolor* A3(2) is controlled by a plasmid, SCP1 (Vivian, 1971; Hopwood *et al.* 1973). We reported previously that treatment with acriflavin or high temperature during the incubation of streptomycetes caused an increased incidence of colonies deficient in aerial mycelium formation, melanoid pigment production, or the production of the antibiotics kasugamycin or aureothricin (Okanishi, Ohta & Umezawa, 1970). Later, we found that production of the antibiotic chloramphenicol in *Streptomyces venezuelae* ISP5230 was also lost with high frequency after treatment with acriflavin. However, the possibility that high temperature or acriflavin might select strains lacking the abilities described above, or act as mutagens, was not eliminated.

This paper describes studies on the sequence of genetic loci and confirmation by genetic mapping of the existence of a plasmid involved in chloramphenicol production in *S. venezuelae* ISP5230.

METHODS

Organisms and media. Mutant strains derived from *Streptomyces venezuelae* ISP5230 were used in the experiments (Table 1). This is a different strain from that used previously (Okanishi *et al.* 1970). All mutant strains were incubated and maintained in ISP medium No. 2 (yeast extract-malt extract agar; Pridham *et al.* 1957). Crosses were carried out on OPY medium, consisting of: oatmeal, 20 g; peptone, 2.0 g; yeast extract (Difco), 0.5 g; K₂HPO₄, 0.5 g; trace element solution (Küster, 1959), 1.0 ml; adenine hydrochloride,

Table 1. *Mutants of Streptomyces venezuelae* ISP5230 employed in mapping experiments

Strain no.	Genotype*	Remarks
SVM1	<i>lys ilv str pro met</i>	—
SVM4	<i>lys ilv str pro met cpp</i>	Derived from SVM1
SVM2	<i>his leu ade</i>	—
SVM3	<i>his leu ade cpp</i>	Derived from SVM2
SVM5	<i>his leu ade cpp mpp</i>	Derived from SVM3

* *lys*, *ilv*, *pro*, *met*, *his*, *leu* and *ade* indicate nutritional requirements for lysine, isoleucine valine, proline, methionine, histidine, leucine, and adenine, respectively; *str* indicates resistance to 100 µg streptomycin/ml; *cpp* and *mpp* indicate inability to produce chloramphenicol and melanoid pigments, respectively. Wild-type alleles are omitted.

10 mg; agar, 20 g; and deionized water to 1 l. Minimal medium (MM) consisted of: glucose, 10 g; glycine, 1.0 g; NH_4NO_3 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g; trace element solution (Okanishi & Gregory, 1970), 1.0 ml; KH_2PO_4 , 2.0 g; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4.0 g; special agar-noble (Difco), 22 g; and deionized water to 1 l. The buffer solution, consisting of KH_2PO_4 and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, was autoclaved separately and added to the medium before cooling. For isolating or characterizing auxotrophic mutants and recombinants, MM was supplemented as follows: individual amino acids, 50 µg/ml; adenine hydrochloride, 10 µg/ml; streptomycin sulphate, 100 µg/ml, or yeast extract (Difco), 2 mg/ml. Chloramphenicol (20 µg/ml) was often added to MM to prevent bacterial contamination. SPN medium for chloramphenicol production consisted of: soluble starch, 20 g; peptone, 5.0 g; NaNO_3 , 2.0 g; KCl, 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g; adenine hydrochloride, 10 mg; KH_2PO_4 , 2.0 g; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4.0 g; agar, 20 g; and deionized water to 1 l. Formation of melanoid pigments was tested on a tyrosine-yeast extract agar medium consisting of (g/l): glucose, 2.0; yeast extract (Difco), 10.0; L-tyrosine, 0.5; NaCl, 5.0; agar, 20; and deionized water to 1 l. Unless otherwise stated, incubation was at 27 °C.

Mutagenic treatment. All auxotrophic and streptomycin-resistant strains were isolated after treating spores with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1 mg/ml) at 27 °C for 80 min in saline (0.5 %, w/v, NaCl) containing 0.05 M-tris-HCl buffer pH 8.4 (Delić, Hopwood & Friend, 1970). Strains lacking production of chloramphenicol or melanoid pigments were obtained by allowing spores to grow in MM containing 2 mg yeast extract and 5 or 10 µg acriflavin/ml as described previously (Okanishi *et al.* 1970).

Assay of antibiotic production. Using the agar-piece method described by Ichikawa *et al.* (1971), the strains to be tested were incubated on agar discs (5 mm deep and 7 mm in diameter) of SPN medium for 5 days. The colonies, together with their agar discs, were transferred to an antibiotic assay plate inoculated with *Escherichia coli* NIHJ and *E. coli* NIHJ-Cf-2 (resistant to chloramphenicol), in a ratio of 1:0.001. After standing for 2 h at 4 °C, they were incubated overnight at 37 °C and the inhibition zones were measured. It was confirmed in advance that production of chloramphenicol on the agar disc corresponded well with that in a liquid medium.

Crossing procedure and determination of recombinant genotype. The crossing technique used in these experiments was virtually identical to that described by Hopwood (1967). Before crossing, each parental strain, purified by re-isolating each clone, was incubated on a slant of OPY medium for 1 to 2 weeks. After sufficient sporulation, spores were harvested into 2 ml of sterile saline containing 0.05 % K_2HPO_4 and 0.05 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The suspension was vigorously shaken on a mixer to break up spore chains, and then passed through cotton wool by means of a hypodermic syringe to eliminate large mycelia or aggregates of

spores. A pair of parental spore suspensions (10^8 /ml) was mixed in a ratio of 1:1 or 1:10, and then samples of about 0.025 ml of the mixture were incubated on slants of OPY medium for 2 weeks. Simultaneously, each parental spore suspension was incubated separately on slants of OPY medium as a control.

Spores formed on the mixed cultures were harvested into 10 to 20 ml of saline, shaken, and filtered as described above. The spore suspension was centrifuged at 10 000 g for 15 min. The spores were resuspended in a small volume of saline and shaken vigorously on a mixer. To select recombinants with all possible pairs of markers, 0.1 ml portions of this spore suspension were spread on to plates containing different supplements. The spore suspension, at suitable dilutions, was also spread on plates of the two media on which each parental strain could grow, to enumerate parental genotypes. After incubating for 4 to 6 days, the colonies grown on the various selective media from a unit volume of the plated suspension were picked with sterile toothpicks, streaked on fresh plates of the selective medium on which they originated and then incubated for 4 days. This streaking procedure was repeated to purify the recombinants or to exclude heteroclones. The recombinant colonies thus obtained were inoculated on to master plates of the selective media on which they originated. After incubating the master plates for 5 days, the colonies were replicated to a series of diagnostic plates to determine their genotypes. The determination was made after incubating for two or three days. The reversion frequency of each parental marker was determined by plating the parental spore suspensions on diagnostic media.

Mapping procedure. To determine the sequence of marker loci, we assumed that the genome of *S. venezuelae* consists of a single circular chromosome. Therefore, only even numbers of crossovers between parental chromosomes are expected to yield haploid recombinants. Recombinants formed by double crossovers should be much more numerous than those arising by quadruple or more crossovers. On this hypothesis, most of the adjacent markers derived from one of the parents should be inherited together.

In practice, crosses were made between parents having many markers; recombinants were selected from a unit volume of the plated suspension by using all possible pairs of markers, except for chloramphenicol and melanoid pigment production, and classified in respect of unselected markers. Recombinants which differed from a parent only in a selected marker were not employed for linkage analysis since they could have included revertants. A sequence of markers was chosen such that markers derived from one parent separated from each other in the smallest number of recombinants. Finally, the sequence of loci was adjusted to minimize the frequency of quadruple crossover (q.c.o.) recombinants.

RESULTS

Origin of chloramphenicol non-producers

The curing of chloramphenicol production by acriflavin treatment was 2 to 5 % in SVM1 and SVM2. The non-producers obtained (*cpp*), after re-isolation, no longer produced chloramphenicol even after incubation in liquid SPN medium for 3 to 6 days, while the producers yielded approximately 30 μ g chloramphenicol/ml. The producers (SVM1 and SVM2) and non-producers (SVM3 and SVM4) did not differ in resistance to chloramphenicol. Growth from spores of all mutants was rather sensitive to chloramphenicol (30 to 50 μ g/ml) on supplemented MM, but their mycelia grew even in a concentration of 200 μ g chloramphenicol/ml. Growth of strains derived from SVM1 (SVM1 or SVM4) was faster than that of strains derived from SVM2 (SVM2, SVM3 and SVM5).

Preliminary genetic experiments

Initially, an attempt was made to select heterokaryons to confirm the existence of a plasmid in *S. venezuelae*, as in *S. scabies* (Gregory & Shyu, 1961; Gregory & Huang, 1964). However, no heterokaryons were found. An attempt was made to study whether or not the *cpp*⁺ character could be transferred to a *cpp* strain in a cross, without transfer of auxotrophic markers. No transfer of *cpp*⁺ was detected in about 1000 progeny with the markers of the *cpp* parent.

When spores from crosses were plated on selective media, a number of heteroclones were usually detectable. Most of them grew as minute colonies on the selective medium. Usually, they scarcely grew when transferred to the same medium, and segregated only a parental type on a complete medium together, sometimes, with a few colonies of recombinant type. These heteroclones were not employed for mapping, because they might have been sensitive to streptomycin if heterozygous for the *str* marker.

Next, numerous crosses were made with the object of constructing a genetic map on the basis of recombination frequencies between markers. Colonies growing on selective plates consisted of haploid recombinants and heteroclones. It was difficult to distinguish the two classes of colony unambiguously in our experimental conditions, even by replica-plating. Furthermore, when equal numbers of parental spores were mixed and incubated, the yields of spores of the two parental types differed significantly, depending on the strains used: strains derived from SVM2 (SVM2, SVM3 and SVM5) yielded only 0.1 to 1.0 % compared with strains derived from SVM1 (SVM1 and SVM4). Therefore, recombination frequencies ranged from 10^{-3} to 10^{-5} when calculated on the basis of the parental yield of strains derived from SVM2, but 10^{-5} to 10^{-7} of the parental yield of strains derived from SVM1. However, nutritional revertants and mutations to streptomycin resistance always appeared at a frequency of 10^{-7} . Therefore, there was a possibility that some of the genotypes differing from a parent by only one marker arose by mutation.

These findings led us to alternative methods of linkage analysis.

Ordering of loci

Crosses were carried out between mutant strains, each having at least three markers (Table 1). Recombinants were selected and purified, and their genotypes in respect of unselected markers were determined by replica-plating, as described in Methods. In these experiments, various recombinants differing from a parent only in a selected marker arose. Certain of these genotypes, which arose with very low frequency, were also detected as revertants with similar frequency in control experiments. In addition, the reversion frequency of each marker varied from cross to cross. These kinds of colonies represented up to about 25 % of the total recombinants obtained. Thus, genotypes differing from a parent by only one marker, as well as heteroclones, were not employed in the determination of the sequence of markers.

Strain SVM1 (*lys*, *ilv*, *str*, *pro*, *met*) was crossed with SVM3 (*his*, *leu*, *ade*, *cpp*) as shown in Table 2. Recombinants were selected from a unit volume of the plated suspension by using all possible pairs of markers, except for *cpp*. The relative numbers of recombinants obtained by each selection, as a percentage of the total recombinants, varied significantly, as follows: *his*⁺/*lys*⁺, 29.7; *ade*⁺/*pro*⁺, 21.4; *ade*⁺/*lys*⁺, 19.0; *str*/*lys*⁺, 6.9; *his*⁺/*met*⁺, 6.6; *his*⁺/*ilv*⁺, 5.9; other pairs, 2.4 to 0.

The sequence of loci was arranged as shown in Table 2. The underlined markers are those derived from the SVM3 parent. The frequency of q.c.o. recombinants, on this marker

Table 2. *Analysis of a cross between strains SVM1 and SVM3 (cross I)*Genotype of SVM1: *his*⁺ *cpp*⁺ *ade*⁺ *str* *leu*⁺ *lys* *met* *ilv* *pro*.Genotype of SVM3: *his* *cpp* *ade* *str*⁺ *leu* *lys*⁺ *met*⁺ *ilv*⁺ *pro*⁺.Selection: all pairs of parental markers (except *cpp*).

Genotypes of recombinants obtained*	No. of recombinants obtained	Quadruple crossover recombinants
<i>his</i> -(<i>cpp</i>)- <i>ade</i> - <i>str</i> - <i>leu</i> - <i>lys</i> - <i>met</i> - <i>ilv</i> - <i>pro</i> -(<i>his</i> -)		
+ (+) + <i>str</i> + + + + +	21	
<i>his</i> (+) + <i>str</i> + <i>lys</i> <i>met</i> <i>ilv</i> +	21	
<i>his</i> (<i>cpp</i>) + <i>str</i> + <i>lys</i> <i>met</i> <i>ilv</i> +	7	
+ (+) + <i>str</i> <i>leu</i> + <i>met</i> <i>ilv</i> <i>pro</i>	19	
+ (<i>cpp</i>) + <i>str</i> <i>leu</i> + <i>met</i> <i>ilv</i> <i>pro</i>	5	‡
<i>his</i> (+) + <i>str</i> + <i>lys</i> <i>met</i> + +	14	
<i>his</i> (<i>cpp</i>) + <i>str</i> + <i>lys</i> <i>met</i> + +	3	
+ (+) + <i>str</i> + <i>lys</i> <i>met</i> + +	6	
+ (<i>cpp</i>) + <i>str</i> + <i>lys</i> <i>met</i> + +	2	‡
+ (+) + <i>str</i> + + + + <i>pro</i>	6	
<i>his</i> (+) <i>ade</i> <i>str</i> + <i>lys</i> <i>met</i> <i>ilv</i> <i>pro</i>	3	‡
<i>his</i> (<i>cpp</i>) <i>ade</i> <i>str</i> + <i>lys</i> <i>met</i> <i>ilv</i> <i>pro</i>	2	
+ (+) + <i>str</i> <i>leu</i> + + <i>ilv</i> <i>pro</i>	2	
+ (<i>cpp</i>) + + <i>leu</i> + <i>met</i> <i>ilv</i> <i>pro</i>	2	‡
+ (+) + + <i>leu</i> + <i>met</i> <i>ilv</i> <i>pro</i>	1	
+ (+) + + <i>leu</i> + + <i>ilv</i> <i>pro</i>	2	
<i>his</i> (<i>cpp</i>) <i>ade</i> + + <i>lys</i> <i>met</i> <i>ilv</i> <i>pro</i>	2	
<i>his</i> (+) <i>ade</i> + + <i>lys</i> <i>met</i> <i>ilv</i> <i>pro</i>	1	‡
<i>his</i> (+) + <i>str</i> + <i>lys</i> + + +	1	
+ (<i>cpp</i>) + <i>str</i> + <i>lys</i> + + +	1	‡
<i>his</i> (+) <i>ade</i> <i>str</i> + <i>lys</i> + + +	1	‡
<i>his</i> (+) <i>ade</i> + + <i>lys</i> + + +	1	‡
+ (+) + + + <i>met</i> <i>ilv</i> <i>pro</i>	1	†
+ (<i>cpp</i>) + + <i>leu</i> + <i>met</i> + <i>pro</i>	1	†
<i>his</i> (+) + <i>str</i> <i>leu</i> + + <i>ilv</i> +	1	†
+ (+) <i>ade</i> <i>str</i> + <i>lys</i> <i>met</i> <i>ilv</i> +	1	†
Total	127	

Minimal quadruple crossover recombinants: 3.2 % for the marker sequence without *cpp*; 15.8 % for the marker sequence with *cpp*. Chloramphenicol production of parental genotypes: all of the 187 spores of the SVM1 parental genotype and none of the 218 spores of SVM3 produced chloramphenicol.

* The underlined markers are those derived from strain SVM3.

† Quadruple crossover recombinants ignoring *cpp*.

‡ Additional q.c.o. recombinants when *cpp* was placed in the sequence minimizing q.c.o.

sequence, was 3.2 % of the 127 recombinants, when *cpp* was disregarded. These q.c.o. recombinants are marked '†' in Table 2. If *ade* was exchanged with *str*, to give the second most likely sequence, the frequency of q.c.o. recombinants increased to 11.8 %. When the *cpp* marker was included, a position between *his* and *ade* minimized the q.c.o. frequency. However, this resulted in a q.c.o. frequency for the whole sequence of 15.8 %, a far larger

Table 3. Analysis of a cross between strains SVM1 and SVM5 (cross II)

Genotype of SVM1: *his*⁺ *mpp*⁺ *ade*⁺ *str* *cpp*⁺ *leu*⁺ *lys* *met* *ilv* *pro*.
 Genotype of SVM5: *his* *mpp* *ade* *str*⁺ *cpp* *leu* *lys*⁺ *met*⁺ *ilv*⁺ *pro*⁺.
 Selection: all pairs of parental markers (except *cpp* and *mpp*).

Genotypes of recombinants obtained*	No. of recombinants obtained	Quadruple crossover recombinants
<i>his</i> -(<i>mpp</i>)- <i>ade</i> - <i>str</i> -(<i>cpp</i>)- <i>leu</i> - <i>lys</i> - <i>met</i> - <i>ilv</i> - <i>pro</i> -(<i>his</i> -)		
+ (+) + <i>str</i> (+) + + + + +	26	
+ (+) + <i>str</i> (+) + + + + <i>pro</i>	17	
+ (<i>mpp</i>) <i>ade</i> + (<i>cpp</i>) <i>leu</i> + + + <i>pro</i>	16	
+ (+) + <i>str</i> (<i>cpp</i>) <i>leu</i> + <i>met</i> <i>ilv</i> <i>pro</i>	8	
+ (+) + <i>str</i> (+) <i>leu</i> + <i>met</i> <i>ilv</i> <i>pro</i>	8	
+ (<i>mpp</i>) + <i>str</i> (<i>cpp</i>) + <i>lys</i> <i>met</i> + +	7	‡, §
+ (+) + <i>str</i> (<i>cpp</i>) + <i>lys</i> <i>met</i> + +	1	‡
+ (+) + <i>str</i> (+) <i>leu</i> + + <i>ilv</i> <i>pro</i>	4	
+ (+) + <i>str</i> (<i>cpp</i>) <i>leu</i> + + <i>ilv</i> <i>pro</i>	4	
+ (+) + + (<i>cpp</i>) <i>leu</i> + <i>met</i> <i>ilv</i> <i>pro</i>	5	
+ (+) + + (+) <i>leu</i> + <i>met</i> <i>ilv</i> <i>pro</i>	4	‡
+ (+) + + (<i>cpp</i>) <i>leu</i> + + <i>ilv</i> <i>pro</i>	4	
+ (+) <i>ade</i> + (<i>cpp</i>) <i>leu</i> + + <i>ilv</i> <i>pro</i>	3	
+ (+) + + (+) <i>leu</i> + + <i>ilv</i> <i>pro</i>	2	‡
+ (+) + <i>str</i> (<i>cpp</i>) + + + + <i>pro</i>	2	‡
<i>his</i> (+) <i>ade</i> <i>str</i> (+) + <i>lys</i> <i>met</i> <i>ilv</i> <i>pro</i>	2	§
+ (+) + + (+) <i>leu</i> + + + +	2	‡
+ (+) + + (+) + + <i>met</i> <i>ilv</i> <i>pro</i>	2	†
+ (+) + + (+) <i>leu</i> + + + <i>pro</i>	1	‡
+ (+) <i>ade</i> + (+) <i>leu</i> + + <i>ilv</i> <i>pro</i>	1	‡
+ (+) + + (<i>cpp</i>) + + <i>met</i> <i>ilv</i> <i>pro</i>	1	†
+ (+) + + (+) + + <i>ilv</i> <i>pro</i>	1	†
+ (+) <i>ade</i> <i>str</i> (<i>cpp</i>) <i>leu</i> + + <i>ilv</i> <i>pro</i>	1	†
+ (+) + <i>str</i> (<i>cpp</i>) <i>leu</i> + <i>met</i> + +	1	†
Total	123	

Minimal quadruple crossover recombinants: 4.9 % for the marker sequence without *cpp* and *mpp*; 21.1 % for the marker sequence with *cpp*; 12.2 % for the marker sequence with *mpp*. Chloramphenicol production of parental genotypes: all of the 168 spores of the SVM1 parental genotype and none of the 163 spores of SVM5 produced chloramphenicol. Melanoid pigment production of parental genotypes: all of the 168 spores of the SVM1 parental genotype and none of the 163 spores of SVM5 produced melanoid pigment.

* The underlined markers are those derived from strain SVM5.

† Quadruple crossover recombinants ignoring *cpp* and *mpp*.

‡ Additional q.c.o. recombinants when *cpp* was placed in the sequence minimizing q.c.o.

§ Additional q.c.o. recombinants when *mpp* was placed in the sequence minimizing q.c.o.

value than the 3.2 % for the sequence excluding *cpp*. In this cross, there was no change in the chloramphenicol production or non-production of the parental genotypes (see legend to Table 2).

A similar cross was carried out between strains SVM1 (*lys, ilv, str, pro, met*) and SVM5 (*his, leu, ade, cpp, mpp*), as shown in Table 3. In this cross, the melanoid pigment production (*mpp*) character was present in addition to the markers used in the earlier experiment. In this cross, recombinants were again selected by using all possible pairs of markers, except *cpp* and *mpp*. Disregarding *cpp* and *mpp*, the same marker sequence was indicated as in the preceding cross. The q.c.o. frequency on this marker sequence, ignoring *cpp* and *mpp*, was 4.9 % of the 123 recombinants obtained. If *his* was exchanged with *ade*, to give the second most likely sequence, the q.c.o. frequency increased to 19.5 %. The best position for *cpp* was between *str* and *leu*, but the q.c.o. frequency, including *cpp*, was 21.1 %. Similarly, the best position for *mpp* was between *his* and *ade*, giving a q.c.o. frequency including *mpp* (but without *cpp*) of 12.2 %. These values of 21.1 and 12.2 % for the marker sequences including *cpp* or *mpp*, were far larger than the 4.9 % value obtained by excluding them. In this cross, the chloramphenicol and melanoid pigment characters of the parental genotypes remained unchanged (see legend to Table 3).

A reciprocal cross for the *cpp* character, for comparison with the above crosses, was performed: the cross between SVM2 (*his, leu, ade*) and SVM4 (*lys, ilv, str, pro, met, cpp*). In this experiment, recombinants were selected only for *his*⁺/*lys*⁺ and the marker sequence was arranged as in Tables 2 and 3 (Table 4). The q.c.o. frequency, ignoring *cpp*, was 3.6 % of the 140 recombinants, and this value was the lowest for any sequence. When *cpp* was placed between *lys* and *met*, to minimize the q.c.o. frequency, the very high value of 15.7 % was obtained. Again, there was no change in the chloramphenicol character of the parental genotypes (see legend to Table 4).

In the above three crosses, the map position of *cpp* giving the lowest q.c.o. frequency varied significantly from cross to cross: between *his* and *ade* in Table 2, between *str* and *leu* in Table 3, and between *lys* and *met* in Table 4. The q.c.o. frequency for all possible map positions of *cpp* in each of the three crosses was calculated (Table 5). The lowest q.c.o. frequency was between 15.7 and 19.5 %, while the lowest q.c.o. frequency excluding *cpp* was between 3.2 and 4.9 %. In cross I, a position for *cpp* between *his* and *ade* gave the lowest q.c.o. value, 15.8 %. However, this sequence resulted in a value of 33.3 % in cross II and 55.7 % in cross III. Thus, a common sequence minimizing the q.c.o. frequency was not revealed.

As another test, each marker was omitted in turn from the markers presented in cross I (Table 2) and cross II (Table 3), and each sequence of markers was adjusted to minimize the frequency of q.c.o. recombinants with or without *cpp*, ignoring *mpp* (Table 6). Each sequence was found to be identical to that provided from cross I or cross II. In cross I, the minimal q.c.o. frequency calculated from this test resulted in a range from 10.2 to 15.8 % with *cpp*, and 1.6 to 2.4 % without *cpp*. The minimum q.c.o. frequency for the sequence including *cpp* was 4.3 to 9.4 times higher than for that without *cpp*. Similar results were obtained in cross II (Table 6). On the other hand, the sequence including *mpp* indicated 4.9 % q.c.o. frequency when *mpp* was placed between *pro* and *ade* (or *ilv* and *his*), if *his* (or *pro*) was omitted. This value was identical to the lowest q.c.o. frequency for the sequence excluding *mpp* and *cpp*.

Table 4. Analysis of a cross between strains SVM2 and SVM4 (cross III)

Genotype of SVM2: *his* *ade* *str*⁺ *leu* *lys*⁺ *cpp*⁺ *met*⁺ *ilv*⁺ *pro*⁺.Genotype of SVM4: *his*⁺ *ade*⁺ *str* *leu*⁺ *lys* *cpp* *met* *ilv* *pro*.Selection: *his*⁺ and *lys*⁺.

Genotypes of recombinants obtained*	No. of recombinants obtained	Quadruple crossover recombinants
<i>his-ade-str-leu-lys-(cpp)-met-ilv-pro-(his-)</i>		
+ + <i>str leu</i> + (<i>cpp</i>) <i>met ilv pro</i>	26	
+ + <i>str leu</i> + (+) <i>met ilv pro</i>	29	
+ + + <i>leu</i> + (<i>cpp</i>)' <i>met ilv pro</i>	8	
+ + + <i>leu</i> + (+) <i>met ilv pro</i>	19	
+ + + <i>leu</i> + (<i>cpp</i>) + <i>ilv pro</i>	8	‡
+ + + <i>leu</i> + (+) + <i>ilv pro</i>	18	
+ <i>ade</i> + <i>leu</i> + (<i>cpp</i>) + <i>ilv pro</i>	4	‡
+ <i>ade</i> + <i>leu</i> + (+) + <i>ilv pro</i>	8	
+ + <i>str leu</i> + (<i>cpp</i>) + <i>ilv pro</i>	4	‡
+ + <i>str leu</i> + (+) + <i>ilv pro</i>	5	
+ <i>ade</i> + <i>leu</i> + (+) + + <i>pro</i>	3	
+ + <i>str</i> + + (<i>cpp</i>) + + +	1	‡
+ + <i>str</i> + + (+) + + +	1	
+ + + <i>leu</i> + (+) + + +	1	
+ + + + (<i>cpp</i>) <i>met ilv pro</i>	2	†
+ + <i>str</i> + + (<i>cpp</i>) <i>met</i> + <i>pro</i>	1	†
+ + <i>str leu</i> + (<i>cpp</i>) <i>met</i> + <i>pro</i>	1	†
+ + <i>str leu</i> + (+) <i>met</i> + <i>pro</i>	1	†
Total	140	

Minimal quadruple crossover recombinants: 3.6 % for the marker sequence without *cpp*; 15.7 % for the marker sequence with *cpp*. Chloramphenicol production of parental genotypes: 408 of the 409 spores of the SVM2 parental genotype and none of the 260 spores of SVM4 produced chloramphenicol.

* The underlined markers are those derived from strain SVM2.

† Quadruple crossover recombinants ignoring *cpp*.

‡ Additional q.c.o. recombinants when *cpp* was placed in the sequence minimizing q.c.o.

Table 5. Frequency of quadruple crossover recombinants for the marker sequences with *cpp* (or *mpp*) between every other pair of markers

Cross*	Total no. of recombinants	Marker placed	Quadruple crossover recombinants for the marker sequence with <i>cpp</i> or <i>mpp</i> (%)										Q.c.o. recombinants for marker sequence without <i>cpp</i> (and <i>mpp</i>) (%)
			<i>his</i>	<i>ade</i>	<i>str</i>	<i>leu</i>	<i>lys</i>	<i>met</i>	<i>ilv</i>	<i>pro</i>	<i>-(his)</i>		
I (SVM1 × SVM3)	127	<i>cpp</i>	15.8	18.9	17.3	35.4	40.2	40.9	46.5	38.6			3.2
II (SVM1 × SVM5)	123	<i>cpp</i>	33.3	23.6	19.5	29.3	54.5	52.9	47.2	39.0			4.9
		<i>mpp</i>	12.2	13.8	28.5	49.6	64.2	43.9	28.5	17.9			
III (SVM2 × SVM4)	140	<i>cpp</i>	55.7	31.4	18.6	39.3	15.7	38.6	60.7	62.1			3.6

* Data for crosses I, II and III were from Tables 2, 3 and 4, respectively.

Table 6. *Minimal q.c.o. frequencies for the best sequences when each marker was omitted in turn, with or without cpp (and mpp)*

Marker omitted	Cross I*			Cross II*		
	Minimum q.c.o. (%)		A/B ratio	Minimum q.c.o. (%)		A/B ratio
	With <i>cpp</i> (A)	Without <i>cpp</i> (B)		With <i>cpp</i> (A)	Without <i>cpp</i> and <i>mpp</i> (B)	
None	15.8	3.2	4.9	21.1	4.9	4.3
<i>his</i>	10.2	2.4	4.3	21.1	4.9	4.3
<i>ade</i>	10.2	2.4	4.3	20.3	4.1	5.0
<i>str</i>	12.6	2.4	5.3	10.6	0.8	13.3
<i>leu</i>	15.0	2.4	6.3	18.7	1.6	11.7
<i>lys</i>	15.0	2.4	6.3	17.9	2.4	7.6
<i>met</i>	15.8	2.4	6.6	20.3	4.1	5.0
<i>ilv</i>	15.0	1.6	9.4	21.1	4.9	4.3
<i>pro</i>	15.0	2.4	6.3	21.1	4.9	4.3

* Data for crosses I and II were from Tables 2 and 3, respectively.

DISCUSSION

In all crosses between strain SVM1 or its derivatives and SVM2 or its derivatives, the yield of parental progeny of the latter type was only 0.1 to 1 % of the former. The recombination frequency in these crosses therefore represented 10^{-3} to 10^{-5} of the minority parental type. This frequency seems to be similar to that in IF $\times$ IF crosses in *S. coelicolor* (Hopwood *et al.* 1973). The minority status of SVM2 strains is attributed to their slow growth on the crossing medium, since their growth and sporulation were not inhibited by SVM1 derivatives, in contrast to the situation in *S. coelicolor* harbouring the SCP1 plasmid (Vivian, 1971).

Another significant finding was that the recombination frequency between *his*⁺ and *lys*⁺ was always higher than between the other markers. However, polarity with respect to donor and recipient behaviour in our crosses is not clear.

In preliminary experiments, it was difficult to distinguish recombinants from heteroclones and to eliminate the possibility that revertants occurred among recombinants differing in only one marker from a parent. We therefore developed a new method for the determination of marker sequences, which allows the genotypes differing from a parent by only one marker to be omitted without invalidating the analysis. This method could generally be applied to other streptomycetes, especially those in which similar difficulties are encountered. The eight markers of *S. venezuelae* were arranged in the sequence *his-ade-str-leu-lys-met-ilv-pro-(his-)* on a circular linkage group, as shown in Tables 2 to 5. By adopting this sequence, the frequency of q.c.o. recombinants was minimized, to 3.2 to 4.9 % in the three crosses described. These values are in good agreement with results in *S. coelicolor* A3(2) (Hopwood *et al.* 1969; Hopwood, Wildermuth & Palmer, 1970; Lomovskaya, Emeljanova & Alikhanian, 1971), *S. rimosus* (Friend & Hopwood, 1971), and *S. glaucescens* (Baumann, Hütter & Hopwood, 1974). These values, 3.2 % in cross I and 4.9 % in cross II, were far lower than those given by the second most likely sequence (11.8 % in cross I and 19.5 % in cross II). Moreover, genotypes differing from a parent by a single marker are explicable by double crossing-over on the basis of all possible sequences, so that the q.c.o. values calculated here are maximum values. The assumed circularity of the map was also indicated by the finding of linkage between every pair of adjacent markers, including *pro* and *his* (Tables 2 to 4). The marker sequence of *S. venezuelae* can tentatively be compared with that of

S. coelicolor A3(2) (Hopwood *et al.* 1973), nearly every *S. venezuelae* marker having a possible counterpart in *S. coelicolor*, as follows: *his* (in *S. venezuelae*) versus *hisD* (in *S. coelicolor*), *ade* versus *adeB*, *str* versus *strA* or *strB*, *leu* versus *leuA*, *lys* versus *lysA*, *met* versus *metA* and *ilv* versus *ilvB*. A *pro* locus has not been identified between *ilvB* and *hisD* in *S. coelicolor* A3(2), but since *proA* occupies a corresponding position on the opposite side of the map, a counterpart for the *pro* locus of *S. venezuelae* may remain to be discovered in *S. coelicolor*.

Evidence for plasmid involvement in chloramphenicol production in *S. venezuelae* was obtained by analysing the segregation of the *cpp* character in crosses. When the *cpp* marker, as well as *mpp*, was placed between each adjacent pair of the other markers, the q.c.o. frequencies for the sequences including *cpp* or *mpp* were significantly higher (15.7 to 62.1 % for *cpp*, 12.2 to 64.2 % for *mpp*) than those for the best sequence excluding *cpp* and *mpp* (3.2 to 4.9 %; Table 5). The location of *cpp* giving the minimum q.c.o. frequency varied from cross to cross. These results indicate that *cpp* and *mpp* are unlinked with the normal chromosomal markers, and presumably each exists on a separate replicon, a plasmid.

Confirmation that the *cpp* marker was in a different class from the standard markers was provided by a further analysis of the data. By omitting each marker in turn, the minimum q.c.o. frequency for every sequence including *cpp* resulted in high values of 10.2 to 21.1 %, while that for every sequence excluding *cpp* and *mpp* showed only 0.8 to 4.9 % (Table 6). This test also supports the conclusion that *cpp* exists on a linkage group different from that bearing the normal chromosomal markers. In this test, however, the marker sequence including or excluding *mpp* resulted in 4.9 % q.c.o., when *mpp* was placed between *pro* and *ade* (or between *ilv* and *his*) and *his* (or *pro*) was omitted. This is the result from only one cross, and should be checked by more crossing experiments.

The argument is weakened by the possibility that two independent *cpp* variants were used in the crosses, one in SVM4 and the other in SVM3 and SVM5, and these might possibly be non-allelic. However, this seems unlikely, since the curing frequency of *cpp*⁺ was always 2 to 5 % in every strain used. Moreover, whenever the different strains lacking chloramphenicol production, obtained by treatment with acriflavin, were employed in crosses (Table 2 or 3 and 4), the *cpp* character behaved differently from the normal chromosomal markers.

The plasmid carrying *cpp* was not transferred to the other partner in the absence of recombination for chromosomal markers, in contrast to the situation for the SCP1 plasmid controlling fertility in *S. coelicolor* A3(2) (Vivian, 1971). Non-transferable plasmids, however, are well known in eubacteria (Novick, 1969).

The role of the plasmid in chloramphenicol biosynthesis is not yet known. However, if plasmid involvement in the production of antibiotics is found to be general, it is possible that the genetic study of such plasmids could lead to new ways of increasing antibiotic yield and the production of two antibiotics in a single strain. It has been found that the ability to produce kasugamycin and aureothricin (Okanishi *et al.* 1970), and probably streptomycin (Okanishi, unpublished observation), is often lost after treatment with acridine dyes.

Coats & Roeser (1971) reported that three different loci affecting the biosynthesis of the antibiotics zorbamycin and zorbonomycins map on the chromosome, together with 14 auxotrophic markers. They obtained their non-producing mutants by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. In contrast, the chloramphenicol production markers used in our experiments were obtained by treatment with acriflavin which is known to be useful for the elimination of bacterial plasmids (Mitsuhashi, Harada & Kameda, 1961).

Gregory & Shyu (1961) concluded that tyrosinase, and therefore melanin production, in

S. scabies is controlled by a plasmid, on the basis of the genetic analysis of heterokaryons. Our experiments also suggest that the production of melanoid pigments in *S. venezuelae* may be controlled by a plasmid.

Note added in proof. After this manuscript had been submitted for publication, Schrempf, Bujard, Hopwood and Goebel (*Journal of Bacteriology*, vol. 121, pp. 416–421, 1975) reported that a plasmid having unknown function was isolated from IF and UF strains of *S. coelicolor* A3(2). Simultaneously, Kirby, Wright and Hopwood (*Nature, London*, vol. 254, pp. 265–267, 1975) showed that both the production of and resistance to an unidentified antibiotic in *S. coelicolor* A3(2) are determined by genes borne on the plasmid SCP1.

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