Rifampicin-resistant Mutants of *Streptomyces coelicolor* A3(2)

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SUMMARY

Studies with whole cells and crude cell-free extracts of *Streptomyces coelicolor* A3(2) indicate that its RNA polymerase, though sensitive to rifampicin, is less so than most bacterial RNA polymerases. Of two other streptomycetes examined, one resembled *S. coelicolor* A3(2) and the other was more like other prokaryotes in respect of rifampicin sensitivity of its RNA polymerase. Three genetic map locations for rifampicin-resistance (*rif*) mutations of *S. coelicolor* A3(2) were determined, one (*rifA*) at 6 o'clock on the conventional linkage map, very close to *strA*, one (*rifB*) at 11 o'clock, between *leuA* and *thiC*, and a third (*rifC*) at 7 o'clock, between *strA* and *pheA*. In representative *rifB* and *C* mutants, RNA synthesis was resistant to rifampicin in whole cells, but not in extracts, whereas in several *rifA* mutants RNA synthesis was equally resistant both in whole cells and in extracts, suggesting that the *rifA* gene may specify part of the RNA polymerase. None of more than 100 *rifA* mutants isolated possessed morphological abnormalities attributable to the *rifA* mutation.

INTRODUCTION

It has previously been pointed out (Chater & Hopwood, 1973) that an investigation of the DNA-dependent RNA polymerase of *Streptomyces coelicolor* A3(2) is pertinent to our studies of differentiation in this morphologically complex prokaryote (Hopwood, Wildermuth & Palmer, 1970; Chater, 1972). One approach to the study of this enzyme is the isolation of mutants resistant to rifampicin, which in other bacteria usually have an altered RNA polymerase (Wehrli & Staehelin, 1971). It is particularly interesting to note that in *Bacillus subtilis* such mutants are often defective in sporulation (Doi, Brown, Rodgers & Hsu, 1970; Sonenshein & Losick, 1970).

The present paper describes the isolation of three kinds of rifampicin-resistant mutants of *Streptomyces coelicolor* and the identification of probable RNA polymerase mutants among them.

METHODS

Organisms. All mutant strains were derived by mutation and recombination from *Streptomyces coelicolor* A3(2) (Hopwood, 1959; *S. violaceoruber* according to Kutzner & Waksman, 1959). They are listed in Table 1, and a linkage map of all the markers employed is given in Fig. 1. In addition, two independently isolated wild-type streptomycetes were used. Our strain 1326 was 'strain 66' of Lomovskaya, Mkrtumian, Gostimskaya & Danilenko (1972), kindly sent by Dr N. D. Lomovskaya, Institute of Genetics and Selection of Microorganisms, Moscow, U.S.S.R.; this strain has been variously classified as *S. coelicolor* or *S. lividans* (Hopwood & Wright, 1973). Our strain 1160 was *S. albus*, obtained in 1954 from the Commonwealth Mycological Institute, Kew, Surrey (their strain 52766).

Chemicals. Rifampicin was a gift from Dr J. Nüesch of Ciba-Geigy A.G., Basle,

Table 1. *Mutant strains of Streptomyces coelicolor* A3(2)

All strains are of NF fertility type except for 311 which is IF (Hopwood, Chater, Dowding & Vivian, 1973). Where *rif* mutants were derived from a sensitive strain after mutagenesis, the strain number of the parent strain is given under 'Source' with the mutagen in brackets (NTG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MOP = 8-methoxypsoralen + near u.v. irradiation). Where strains were derived by recombination, the parent strains are indicated under 'Source'.

Strain no.	Genotype	Source
311	<i>hisA1 leuA1 strA1</i>	D. A. Hopwood
A321	<i>uraA1</i>	A. Vivian
774	<i>argA1 hisC9 proA1 strA1</i>	D. A. Hopwood
782	<i>argA1 cysC3 hisA1 mthB2 strA1</i>	D. A. Hopwood
876	<i>argA1 cysC3 hisC9 pheA1 proA1 strA1</i>	D. A. Hopwood
957	<i>argA1 cysC3 pheA1 strA1 thiC2</i>	D. A. Hopwood
J222	<i>rifA101 uraA1</i>	A321(NTG)
J223	<i>cysC3 rifA101</i>	J222 × 782
J229	<i>rifB103 uraA1</i>	A321(NTG)
J245	<i>rifB109 uraA1</i>	A321(MOP)
J259	<i>rifC114 uraA1</i>	A321(MOP)
J264	<i>rifA118 uraA1</i>	A321(MOP)
J271	<i>cysC3 pheA1 proA1 rifB103 strA1</i>	J229 × 876
J301	<i>pheA1 rifA114</i>	J259 × 876
J317	<i>pheA1 proA1 rifB109 strA1</i>	J245 × 876
J335	<i>hisC9 rifB103</i>	J229 × 774
J365	<i>argA1 cysC3 hisC9 pheA1 proA1 rifA122 strA1</i>	876(NTG)
J370	<i>argA1 cysC3 hisC9 pheA1 proA1 rifA124 strA1</i>	876(NTG)
J372	<i>pheA1 rifA124 strA1</i>	J370 × A321
J373	<i>pheA1 proA1 rifB125 strA1</i>	J386 × A321
J386	<i>argA1 cysC3 hisC9 pheA1 proA1 rifB125 strA1</i>	876(NTG)
J398	<i>pheA1 rifA122 strA1</i>	J365 × A321
J515	<i>rifA126</i>	A3(2)(MOP)

Switzerland, and streptolydigin was a gift from Dr G. B. Whitfield of the Upjohn Co., Kalamazoo, Michigan, U.S.A.; unlabelled ribonucleoside triphosphates were obtained from P-L Biochemicals, Milwaukee, Wisconsin, U.S.A.; [³H]UTP (13 Ci/mmol; 0.5 mCi/ml) and [³H]uridine (5.5 Ci/mmol; 1 mCi/ml) from the Radiochemical Centre, Amersham, Buckinghamshire; dithiothreitol from B.D.H. Biochemicals, Poole, Dorset; calf thymus DNA from Worthington Biochemicals, Freehold, New Jersey, U.S.A.; and egg-white lysozyme (grade I) from Sigma, London.

Methods of culture. Minimal (MM) and complete (CM) agar media, supplements, and general methods of culture were as described by Hopwood (1967). Rifampicin-supplemented plates were poured after the addition of a freshly prepared solution of rifampicin in methanol to molten agar medium cooled to 50 °C. Final concentrations were 50 to 200 µg/ml for the isolation of mutants, and 25 or 50 µg/ml for classification of recombinants by replica plating. Mycelium for the preparation of cell-free extracts was grown in liquid yeast-extract-malt-extract medium (Townsend, Wright & Hopwood, 1971) containing 33 % (w/v) sucrose and 0.5 % (w/v) MgCl₂. The medium was dispensed in 400 to 500 ml volumes in 2 l Erlenmeyer flasks. Each flask was inoculated with 10⁹ to 10¹⁰ spores and incubated for 24 to 48 h in a rotary shaker at about 200 rev./min. Mycelium was harvested by centrifugation, washed once with distilled water, and stored frozen at -20 °C. Up to 5 g (packed wet wt) mycelium was obtained from each flask.

Isolation of rifampicin-resistant mutants. Spore suspensions were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Delić, Hopwood & Friend, 1970) or with 8-methoxypsoralen

Assay of RNA synthesis in whole cells. Glass-fibre filters (2.5 cm; Whatman, GF/C) were cut into quadrants and laid on an appropriately supplemented MM plate. To each quadrant was added 0.01 ml spore suspension (about 10^7 spores). After 18 h incubation the quadrants

were transferred to a dish containing a shallow layer of pre-incubation medium (liquid minimal medium containing 0.5 % (w/v) glucose, uridine (2 $\mu\text{g}/\text{ml}$) and substances required for the growth of auxotrophs) and incubated at 30 °C for 1 h before transfer to assay medium of the same composition as pre-incubation medium, but containing [^3H]uridine (1 $\mu\text{Ci}/\mu\text{mol}$, 2 $\mu\text{g}/\text{ml}$). The assay medium formed a shallow layer in a dish, and was kept at 30 °C. Where zero time samples were required, quadrants were dipped transiently into the assay medium and then dropped into 5 ml ice-cold 5 % (w/v) trichloroacetic acid (TCA). Further quadrants were then placed in the assay medium and removed to 5 % TCA after appropriate times of incubation. Samples were washed in two 100 ml volumes of 2 % TCA and in one 50 ml volume of ethanol before drying at 80 °C. The dry samples were added to scintillation vials containing 5 ml scintillation fluid [0.5 % (w/v) butyl PBD (Ciba) in toluene], and their radioactivity measured in a Beckman LS-200B liquid scintillation system.

Preparation of crude cell-free extracts. Mycelium harvested from yeast extract-malt extract medium supplemented with 33 % (w/v) sucrose and 0.5 % (w/v) MgCl_2 has increased sensitivity to lysozyme (Townsend, 1973). To prepare extracts such material was suspended in its own weight of extraction buffer (10 mM-tris-HCl, pH 8.3, at 4 °C; 10 mM- MgCl_2 ; 0.1 mM-EDTA, pH 7.0; 0.1 mM-dithiothreitol; 15 % v/v, glycerol) and incubated for 15 min at 30 °C with lysozyme (2.5 mg/ml). After cooling in ice, the suspension was given brief ultrasonic treatment to complete lysis of the weakened cells. Extracts were cleared by low-speed centrifugation, then centrifuged at 78 000 g at 4 °C for 90 min to remove ribosomes. Such 'crude extracts' were stored at -20 °C with little loss of activity over several weeks.

RNA polymerase assay. The reaction mixture comprised: 60 mM-tris-HCl, pH 7.9, at 30 °C; 10 mM- MgCl_2 ; 0.1 mM-EDTA; 50 mM- NH_4Cl ; 0.15 mM-ATP, CTP, GTP and [^3H]UTP (17 mCi/mmol); 2 mM-spermidine; 0.375 mM-potassium phosphate buffer, pH 7.3; 0.1 mM-dithiothreitol; and calf thymus DNA at 0.05 mg/ml. The total volume of the reaction mixture varied from 0.1 to 0.3 ml, as stated. After addition of crude extract, the mixture was incubated at 30 °C for 10 min and the reaction stopped by placing in ice. Three ml ice-cold 5 % TCA containing 10 mM-sodium pyrophosphate was added. After 15 min the insoluble material was collected on a glass-fibre filter presoaked in 2 % TCA + 10 mM-sodium pyrophosphate, and washed with eight 1.5 ml volumes of 2 % TCA + 10 mM-sodium pyrophosphate and finally with 3 ml ethanol. The filter was dried at 80 °C and its radioactivity determined as described above.

RESULTS

*The effect of rifampicin on RNA synthesis by whole cells of *Streptomyces coelicolor* A3(2)*

RNA synthesis by whole cells was assayed by measuring the incorporation of [^3H]uridine into TCA-insoluble material. Although such measurements are relatively simple with single celled organisms, where homogeneous cell suspensions can be used, the mycelial growth of *Streptomyces coelicolor* and its tendency to form clumps necessitated the development of a special technique. Pieces of glass-fibre filters carrying approximately similar amounts of young mycelium were prepared as described in Methods. These were incubated for various time intervals in an assay medium containing [^3H]uridine, then placed in ice-cold 5 % TCA, washed, and the incorporation of ^3H into TCA-insoluble material estimated. Although the method was rather crude, it was sufficiently reliable to be used in preliminary studies of the effect of rifampicin on RNA synthesis. Fig. 2 shows that RNA synthesis was rapidly and

Fig. 2. RNA synthesis by whole cells of *Streptomyces coelicolor* in the presence of rifampicin and streptolydigin. The assays were carried out with glass-fibre quadrants carrying young mycelium as described in Methods. Each point on the graph corresponds to the mean amount of [³H]uridine incorporated into TCA-insoluble material by duplicate samples incubated for the stated time in [³H]uridine-containing assay medium. ○, Control (no antibiotic); ●, rifampicin (100 µg/ml) at 5 min; ▲, streptolydigin (100 µg/ml) at 5 min. The arrow marks the point when antibiotic was added.

Table 2. The effect of different rifampicin concentrations on RNA synthesis by whole cells of *Streptomyces coelicolor*

The assays were carried out with filter quadrants as described in Methods, except that the specific activity of uridine was 0.1 µCi/µmol. Samples were pre-incubated in non-radioactive medium with rifampicin for 5 min before being transferred to assay medium containing [³H]uridine and rifampicin. The reaction was stopped at 5 min. The means for duplicate samples in three replicate experiments are given.

Counts/min in sample without rifampicin	Residual activity at different concentrations of rifampicin (%)			
	2 µg/ml	5 µg/ml	20 µg/ml	50 µg/ml
452	59	30	18	24
327	40	11	8	5
472	56	29	18	—
Mean	52	23	15	15

severely inhibited by the antibiotic at 100 µg/ml, and that streptolydigin, another inhibitor of bacterial RNA polymerase (Siddhikol, Erbstoesser & Weisblum, 1969), had a similar effect also at 100 µg/ml. The amount of inhibition caused by different rifampicin concentrations is given in Table 2. At 2 µg/ml the inhibition was about 50 %, and even at much higher concentrations inhibition was not complete. In later experiments (Table 10) even less inhibition was observed.

Table 3. *The effect of GTP and streptolydigin on the RNA polymerase activity of an extract of S. coelicolor A3(2)*

Details of the assay are given in Methods. The total assay volume was 0.225 ml. Results are the mean values of duplicate assays.

Additions and omissions	Counts/min
None	1022
– GTP	208
+ Streptolydigin (20 µg/ml)	210
– GTP, + streptolydigin	183

Table 4. *Inhibition by rifampicin of RNA polymerase activity in crude extracts of Streptomyces coelicolor A3(2)*

Extract (0.025 ml) was incubated with 0.025 ml rifampicin or streptolydigin solution at four times the stated concentration, or with 0.025 ml 5% (v/v), methanol/water at 30 °C for 5 min. The components of the assay were then added to give a final volume of 0.1 ml, and incubation continued for a further 10 min, when the reaction was stopped. For further details see Methods. Percentage residual activities were calculated after subtraction of the activity obtained in the presence of streptolydigin (20 µg/ml) (see text).

Counts/min		Residual activity at different rifampicin concentrations (%)		
– Rifampicin	+ Streptolydigin	1 µg/ml	20 µg/ml	100 µg/ml
1213	214	69	31	22

The effect of rifampicin on RNA polymerase activity in crude cell-free extracts of different wild-type streptomycetes

Using the assay system described in Methods, the incorporation of tritium into TCA-insoluble material in the presence of a crude cell-free extract of *Streptomyces coelicolor* A3(2) was linear with time up to 15 min at 30 °C. At 37 °C the reaction was faster but shorter-lived, so all the experiments reported in this paper were done at 30 °C.

A somewhat variable GTP-independent component was found in some extracts, and it was necessary to know whether this was attributable to the major RNA polymerase enzyme or was independent of it. Experiments with purified RNA polymerase (K. F. Chater, unpublished) showed that the GTP-independent activity was lost during purification, indicating that it was independent of the RNA polymerase. Since a further experiment with the purified GTP-dependent enzyme also showed that streptolydigin at 20 µg/ml gave almost complete inhibition of activity, it was of interest to find that activity of the GTP-independent component found in crude extracts was scarcely, if at all, inhibited by streptolydigin at 20 µg/ml (Table 3). This observation led to the use in all experiments of a duplicate control assay containing streptolydigin at 20 µg/ml, which was equivalent to a control lacking GTP. The mean value obtained was subtracted from all readings to give a better estimate of the RNA polymerase activity.

The effect of different concentrations of rifampicin on RNA polymerase activity of *Streptomyces coelicolor* A3(2) is given in Table 4. The results in Tables 2 and 4 indicated that the effect of rifampicin was quantitatively and qualitatively similar *in vivo* and *in vitro*, and that the RNA polymerase of strain A3(2) was far less sensitive to rifampicin than is that of other prokaryotes (Wehrli & Staehelin, 1971). This raised the question, is strain A3(2) typical of streptomycetes in this respect? Extracts of two other streptomycetes were therefore

Table 5. Inhibition by rifampicin of RNA polymerase activity in crude extracts of three streptomycetes

Strain	Counts/min		Residual activity (%) with two rifampicin concentrations	
	- Rifampicin	+ Streptolydigin	1 µg/ml	20 µg/ml
A3(2) (<i>S. coelicolor</i>)	1008	97	75	48
1326 (<i>S. lividans</i>)	1267	188	83	46
1160 (<i>S. albus</i>)	1045	220	1	0

The procedure and calculations were as in Table 4 except that all quantities in the assay mixture were doubled, giving a final volume of 0.2 ml, and the extracts were pre-incubated for only 2 min with rifampicin.

Table 6. Preliminary mapping of the *rif-101* mutation

Strain 782 (outer circle) was crossed with the rifampicin-resistant strain J222 (inner circle) and selection was made for recombinants inheriting *arg*⁺ and *ura*⁺ (triangles). The frequency of each allele among the progeny is given on the diagram, together with alternative locations (arrows) for *rif-101* based on its allele frequency. Examination of the pattern of recombination shows absence of recombination between *strA1* and *rif-101* (giving *strA1rif-101* or *strA*⁺*rif*⁺ recombinants) and independent assortment of *hisA1* and *rif-101* indicating a location of *rif-101* close to *strA*.

made and the effects of rifampicin on their RNA polymerase activity were measured (Table 5). It turned out that strain 1326 was very similar to A3(2), whereas strain 1160, which in other respects also is less similar to A3(2) than is 1326, differed considerably: its activity was completely inhibited by rifampicin at 1 µg/ml, at which concentration the A3(2) and 1326 activities were only slightly affected (25 % and 17 %, respectively). It is unlikely that the results with the 1160 extract can be partially attributed to a lower sensitivity of its enzyme to streptolydigin than was found for A3(2) (thus causing a falsely high estimate of RNA polymerase-independent ³H incorporation), or to sensitivity to rifampicin of the streptolydigin-

resistant component: the inhibition obtained with rifampicin or streptolydigin at either 1 $\mu\text{g/ml}$ or 20 $\mu\text{g/ml}$ was approximately the same, and no reduction of the streptolydigin-resistant activity was observed in the presence of rifampicin (K. F. Chater, unpublished).

Rifampicin-resistant mutants

In the first isolation, a large number of rifampicin-resistant (*rif*) mutants were isolated from strain A321 as papillae on the surface of a confluent layer of growth after 4 days' incubation on plates containing 100 μg of rifampicin/ml. Four of these were crossed with strain 782, and all gave similar results. In the example shown in Table 6 the allele frequencies located *rif-101* either between *hisA1* and *uraA1*, or very close to *strA1*. Since *rif-101* segregated independently of *hisA1* ($\chi^2 = 1.1$) but not of *strA1* ($\chi^2 = 104$), it was concluded that *rif-101* was located very close to *strA1*, identifying the *rifA* locus.

The very close linkage of *rif-101* and *strA1* made it easy to carry out a rapid method of identifying *rif* mutations located elsewhere on the linkage map. Up to 24 mutants per plate were inoculated as patches over a template and allowed to grow and sporulate, before replica plating to CM spread with spores of strain 782 (see Table 6 for diagram of marker arrangements), thus giving 24 crosses on a single plate. After 4 days' incubation, the plates were replica plated to appropriately supplemented MM selecting *strA1* from strain 782 and the diametrically situated *hisA*⁺ from the rifampicin-resistant strains. Clearly, recombinants arising from crosses with strains in which the *rif* mutation was closely linked to *strA* would nearly all have received the *rif*⁺ allele of strain 782, and would therefore be rifampicin-sensitive. On the other hand, where the rifampicin-resistant parent strain had a *rif* mutation not very closely linked to *strA*, a significant proportion of streptomycin-resistant recombinants would also be rifampicin-resistant. The selective plates containing streptomycin were therefore incubated for 4 days and replica plated to similar plates containing rifampicin. Fifty-nine rifampicin-resistant mutants from the first mutant isolation were tested by this method. Nearly all possessed *rif* mutations closely linked to *strA*; however, two gave a high frequency of *strA rif* recombinants, indicating fairly close linkage between *hisA1* and the *rif* mutation. Similar rapid mapping was done with 54 mutants from four subsequent isolations, in which different starting strains and post-mutagenic treatments were employed. Seventeen more examples were found of mutations located near to *hisA*, together with a single example of a third type of mutant giving intermediate frequencies of *strA rif* recombinants on the plate test. *hisA*-linked mutations were only found among mutants which had been isolated on lower concentrations (50 to 100 $\mu\text{g/ml}$) of rifampicin, and the third type of mutant was also isolated on 50 $\mu\text{g/ml}$. Thus, all *rif* mutants isolated on a high concentration (200 $\mu\text{g/ml}$) of rifampicin possessed *strA*-linked mutations. The mutant spectrum was not related to the mutagen employed or to the kind of post-mutagenic treatment.

More precise genetic mapping of hisA-linked rif mutations

The independently isolated *hisA*-linked *rif* mutations were shown in preliminary crosses with strain 782 to be located close to, and anticlockwise of, *hisA*; in the example given in Table 7, a choice between the two alternative locations suggested by consideration of the allele frequency is easily made, because the segregation of *rif-103* was independent of *cysC3* ($\chi^2 = 0.5$), but not of *hisA1* ($\chi^2 = 58$).

Further crosses of these and two other mutants with strain 876 (data not given) indicated a map location between *proA1* and *hisA1*; a similar result was also obtained with crosses of strain A321 with two resistant derivatives of strain 876. Since all the data were similar, it

Table 7. Preliminary genetic mapping of the *rif-103* mutation

Strain 782 (outer circle) was crossed with the rifampicin-resistant strain J229 (inner circle) and selection was made for recombinants inheriting *uraA*⁺ and *argA*⁺ (triangles). The frequency of each allele among the progeny is given on the diagram, together with alternative locations (arrows) for *rif-103* based on its allele frequency. The tabulations show clearly that *rif-103* segregated independently of *cysC3* but not of *hisA1*, indicating the location between *hisA* and *uraA*.

seemed probable that only one locus was involved, so two representative mutations were selected for use in further mapping studies to determine its location. Recombinants had been obtained from the crosses with 876 having the *rif* mutations in combination with *proA1*. These were crossed with strains 311 and 957, to locate the *rif* mutations with respect to the *leuA* and *thiC* genes (Table 8). It was clear that both mutations were situated anti-clockwise of *thiC*, and that *rif-103* was clockwise of *leuA*. The data for the location of *rif-109* with respect to *leuA* were less clear, and there were relatively few *leuA rif* recombinants. A cross between *hisC9 rif-103* and *proA1 rif-109* recombinants gave 96 resistant colonies out of 98 scored, a result showing that *rif-109* was situated either clockwise of *rif-103* (when the two sensitive recombinants would have resulted from multiple crossing over) or anti-clockwise of *rif-103* but very close to it. Consideration of this result leads to the conclusion that the *rif-109* mutation, like *rif-103*, is situated between *leuA* and *thiC*. In the absence of more detailed information, all the *hisA*-linked *rif* mutations obtained so far are considered to be mutations in one gene, termed *rifB*.

Genetic mapping of the *rif-114* mutation

The *rif* mutation in strain J259 (*uraA1 rif-114*) was recognized as being distinct from *rifA* and *rifB* mutations by rapid mapping tests. In a preliminary cross with strain 876 (Table 9) the allele frequency gave the location of the mutation as either between *pheA* and *strA*, or between *uraA* and *proA*; analysis of the segregation pattern showed that the former location was correct, as the segregation of *rif-114* was almost independent of that of *proA1* ($\chi^2 =$

Table 8. *More precise location of rifB mutations*

Strain no.	Diagram of cross	Regions of crossing-over	Relevant genotype	Frequency among recombinants examined with	
				<i>rif-103</i>	<i>rif-109</i>
J271 or J317		1	<i>rif</i>	23	47
		2	+	23	22
		3	<i>thiC3</i>	54	33
957		1, 2, 3	<i>rif thiC3</i>	0	0
				100	102
J271 or J317		1	<i>rif</i>	25	22
		2	<i>leuA1 rif</i>	41	6
		3	<i>leuA1</i>	33	34
311		1, 2, 3	+	1	5
				100	67

Strains J271 and J317 were crossed with strains 957 and 311, with the selections indicated by triangles in the diagrams. For further explanation, see text.

5-6) but not of *pheA1* ($\chi^2_1 = 63$). The data of Table 9 clearly show that *rif-114* is not close to *strA*, to which *rifA* mutations are very closely linked. To confirm that *rif-114* was indeed distinct from mutations at the *rifA* locus a cross of strain J301 (*pheA1rif-114*) with J223 (*cysC3rifA101*) was made, with selection for prototrophic recombinants. Sixty recombinants out of 102 tested were sensitive to rifampicin, confirming that *rif-114* was not a *rifA* allele and was located clockwise of *rifA*. It was taken to be the unique representative of a third rifampicin-resistance locus, *rifC*.

The effect of rifampicin on RNA synthesis by whole cells and extracts of rifampicin-resistant mutants

Representative mutants possessing *rifA101*, *rifB125* and *rifC114* were initially examined. The results for the *rifB* and *C* mutants with whole cells differed strikingly from those with extracts: incorporation of [³H]uridine into whole cells was scarcely affected by rifampicin (up to 20 μ g/ml) whereas in extracts the pattern of rifampicin-sensitivity of RNA polymerase was much the same as in the wild type (Table 10). From these results it seemed probable that the *rifB* and *C* mutants owed their rifampicin resistance to a reduced ability of the cell to take up the antibiotic. In contrast, the results for the *rifA101* mutant were closely similar in whole cells and extracts, in both cases indicating little difference from the wild type. To clarify this result, several other *rifA* mutants which had been isolated on relatively high rifampicin concentrations (up to 200 μ g/ml) were tested (Table 10), and their RNA polymerase activities were found to have greatly increased resistance to rifampicin compared with the wild type. There was even some evidence for stimulation of activity by rifampicin with some extracts: no experiments have been made to determine the nature of this stimulation. Overall, the results in Table 10 constitute strong preliminary evidence that *rifA* mutants possess RNA polymerase altered in its rifampicin sensitivity.

Table 9. Preliminary genetic mapping of the *rif-114* mutation

Strain 876 (outer circle) was crossed with strain 1259 (inner circle) and selection was made for recombinants inheriting *ura*⁺ and *arg*⁺ (triangles). The frequency of each allele among the progeny is given on the diagram, together with alternative locations (arrows) for *rif-114* based on its allele frequency. The tabulations show that segregation of *rif-114* is much more dependent on that of *pheA1* than of *proA1*, indicating the location between *pheA* and *strA*.

The morphology of *rif* mutants

In the course of this work about a hundred mutants presumed on the basis of genetic mapping to be *rifA* have been isolated. In one isolation experiment many mutants were devoid of aerial mycelium ('bald'), and three other 'bald' *rifA* mutants were obtained in separate isolations. However, despite this apparently high rate of coincidence of the *rifA* mutation and morphological abnormality, in each case the bald mutation segregated from the *rifA* mutation in crosses. Thus we have no evidence of a morphological effect resulting from an altered RNA polymerase, in contrast to the situation described for *Bacillus subtilis* (Sonenshein & Losick, 1970; Doi *et al.* 1970).

DISCUSSION

The results in this paper have shown that streptomycetes, like other prokaryotes, possess rifampicin-sensitive RNA polymerase, though the degree of sensitivity is relatively low in some wild-type strains: it was found that while the *Streptomyces albus* (strain 1160) enzyme was almost completely inhibited by rifampicin concentrations as low as 0.01 µg/ml (Table 5; and K. F. Chater, unpublished), a concentration of the order of 10 µg/ml was required to give 50 % inhibition of the enzyme of two more closely related strains, *S. coelicolor* A3(2) and

Table 10. *The effect of rifampicin on RNA synthesis by whole cells and extracts of rifampicin-resistant mutants of Streptomyces coelicolor* A3(2)

Determinations with whole cells were made by the filter quadrant method as described in Table 2 and Methods, using a 10 min incubation period and [³H]uridine at 1 μ Ci/ μ mol. Determinations and calculations with extracts were done as described in Table 4 and Methods, reaction volumes varying from 0.1 to 0.3 ml.

Strain	rif mutation	Whole cells or extract	Total activity with no inhibitor (counts/min)	Activity with streptolydigin (counts/min)	Residual activity (%) at various concentrations of rifampicin		
					5 μ g/ml	20 μ g/ml	100 μ g/ml
A3(2)	—	Whole cells	5109	—	65	39	—
A3(2)	—	Extract	458	115	58	38	22
J223 }	rifA101	{ Whole cells	4885	—	56	39	—
J222 }		{ Extract	671	166	43	32	13
J386 }	rifB125	{ Whole cells	2735	—	96	72	—
J373 }		{ Extract	1176	498	39	23	—
J301 }	rifC114	{ Whole cells	3731	—	89	58	—
J259 }		{ Extract	713	51*	30	16	—
J372	rifA124	Whole cells	4075	—	69	77	—
		Extract	541	113	—	150	120
J515	rifA126	Whole cells	3147	—	108	97	—
		Extract	380	201	—	91	71
J264	rifA118	Extract	264	117	—	77	90
J398	rifA122	Extract	452	105	—	113	107

* For strain J259 the streptolydigin control was replaced by a control lacking GTP, which gave 51 counts/min.

S. lividans (strain 1326). Since the RNA polymerase activity of A3(2) has a similar profile of rifampicin sensitivity in whole cells, in crude extracts, and in highly purified preparations (Table 10; and K. F. Chater, unpublished), the relatively low rifampicin-sensitivity cannot easily be accounted for either by pre-attachment of the enzyme to endogenous DNA to form a rifampicin-resistant complex (Sippel & Hartmann, 1970), or by rifampicin breakdown or removal by factors present in extracts.

The isolation of mutants which appear to have decreased permeability to rifampicin has been described for *Escherichia coli* (Patterson, Weinstein, Marshall & Gillespie, 1971) and *Bacillus subtilis* (Brown & Doi, 1970). In the case of *E. coli*, the Rif-r (permeability) mutations are closely linked, but complementary, to *rifA* mutants having an altered RNA polymerase (Marshall & Gillespie, 1972). In *Streptomyces coelicolor* there are two loci, *rifB* and *rifC*, in which mutations seem to result in reduced permeability of cells to rifampicin. These are not closely linked to each other, or to the *rifA* locus. The evidence that *rifA* specifies the rifampicin-sensitive component of RNA polymerase is supported by recent experiments (K. F. Chater, unpublished) in which RNA polymerase of a *rifA* mutant has been purified about 40-fold through to the DEAE cellulose step of Burgess (1969): such enzyme retained its resistance to rifampicin.

Close linkage of the gene for rifampicin-resistant RNA polymerase with loci specifying antibiotic-resistant ribosomes has been noted in *Bacillus subtilis* (Harford & Sueoka, 1970). Moreover, conservation of this gene arrangement and of the nucleotide sequences involved are indicated by the finding that the rate of transformation between various

Bacillus species is much higher for rifampicin- and erythromycin-resistance genes than for auxotrophic markers, and that co-transformation is usually detectable (Harford & Mergeay, 1973). It is thus interesting to find that *strA* and *rifA* are also very closely linked in *Streptomyces coelicolor*; one would like to know, in the context of our understanding of the meaning of the arrangement of genes on the bacterial chromosome, whether they are also rather closely linked to the origin of replication in *S. coelicolor* as has been found in *B. subtilis* (Harford & Sueoka, 1970), *Escherichia coli* (Taylor, 1970; Bird, Louarn, Martuscelli & Caro, 1972) and *Salmonella typhimurium* (Sanderson, 1970; Nishioka & Eisenstark, 1970).

Morphological abnormalities attributable to *rifA* mutations were not found in *Streptomyces coelicolor*, though they are frequently encountered in *Bacillus subtilis* (Sonenshein & Losick, 1970; Doi *et al.* 1970). This does not of course exclude the possibility that changes of RNA polymerase structure and/or specificity are involved in differentiation in *S. coelicolor*, though it suggests that if any such changes do occur they probably involve regions of the enzyme distinct from the rifampicin binding site.

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