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Novel pleiotropic effect of rifampicin resistance mutation in a *Micromonospora* sp.

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SUMMARY

Rifampicin-resistant mutants have been isolated from a *Micromonospora* sp. In one of these, rifampicin failed to inhibit [^{3}H]UTP incorporation in osmotically shocked cells; consequently, resistance was probably not due to the alteration of rifampicin permeability. Parallel to the rifampicin resistance there was a substantial increase in the novobiocin sensitivity of the mutants. Rifampicin-sensitive revertants exhibited their original novobiocin sensitivity. At the same time there was no increase in their sensitivity towards coumermycin A_1, an agent of related structure and activity. The possible mechanism for this pleiotropy is discussed.

1. INTRODUCTION

Rifampicin binds to the β-subunit of DNA-dependent RNA polymerase, specifically inhibiting the initiation of RNA synthesis, and thereby blocking bacterial cell growth. Since the conformation of RNA polymerase plays an active role in promoter selection, it is not surprising that mutations inducing rifampicin resistance can also change the specificity of promoter selection by the enzyme. This results in the well known pleiotropic effect of some rifampicin resistant mutations. In the case of *Bacillus subtilis* these may induce sporulation defects (Leighton, 1973; Sonenshein & Losick, 1970; Sonenshein *et al.* 1974; Sumida-Yasmumoto & Doi, 1977), or changes in spore morphology (Doi *et al.* 1970; Korch & Doi, 1971). Ikeuchi, Babasaki & Kurahashi (1979) have observed phenotypic changes in *spoOC-spoOA*; Ryu (1978), loss of activity of glutamic acid synthetase, an enzyme of nitrogen metabolism; and Snyder (1972), inhibition of the growth of an *Escherichia coli* phage. Hong, Smith & Ames (1971) noted reduction in the frequency of lysogenization in *Salmonella typhimurium*, and Grodzicker, Arditti & Eisen (1972) noted the same phenomenon in *E. coli*. In *E. coli*, *dnaA* mutations may also be suppressed (Bagdasarian *et al.* 1977).

A further factor regulating the binding of RNA polymerase to DNA is the degree of supercoiling of the template. *In vivo* DNA gyrase is believed to be responsible

for the supercoiled state of the genome. Consequently all antibiotics (e.g. novobiocin, and coumermycin A_1) interacting with DNA gyrase are likely to affect both DNA and RNA synthesis.

In the present study we investigated whether rifampicin resistance mutations in two different *Micromonospora* strains could change their sensitivity to novobiocin. These results were presented in preliminary form at the International Symposium on Antibiotics, Weimar (Gadó *et al.* 1979).

2. MATERIALS AND METHODS

(i) *Organisms*

Micromonospora strain S1/109 was isolated from river mud and produces an antibiotic complex with sisomicin as major component. *Micromonospora echinospora* ATCC 15837 produces the gentamicin-C complex.

(ii) *Materials*

Oxytetracycline, streptomycin and bacitracin were supplied by Chinoin; penicillin G by Biogal; acridine orange by Reanal; viomycin, mithramycin, actinomycin-D, neomycin, D-cycloserine, erythromycin and paromomycin by the Institute for Drug Research, novobiocin by Sigma, chloramphenicol, mitomycin C and rifampicin by Serva. We should like to express our thanks to Dr Julius Berger (Hoffman-LaRoche) for the sample of coumermycin A_1.

(iii) *Media*

Complete-agar: 3 g meat extract, 5 g tryptone, 1 g glucose, 24 g water-soluble starch, 5 g yeast extract, 2 g $CaCO_3$, 0·2 g $FeSO_4.7H_2O$, 2 g $MgSO_4.7H_2O$, 15 g agar in 1 l. tap water, pH = 7·0.

TS medium: 5 g tryptone, 10 g soymeal, 1 g glucose, 24 g water-soluble starch, 2 g $CaCO_3$ in 1 l tap water, pH = 7·0.

CGT medium: 12 g Tris base, 1 g NH_4Cl, 0·2 g $MgCl_2.6H_2O$, 0·058 g NaCl, 0·3 g $Na_2SO_4.10H_2O$, one drop of 1 % $FeCl_2$ in 1 l distilled water, pH adjusted with HCl to 7·4. Following sterilization 0·2 % glucose and 0·2 % Difco casamino acids, sterilized separately, were added.

(iv) Rif^r *mutants*

The selection of spontaneous rifampicin resistant (*rif*^{r}) mutants was carried out on complete-agar containing 10 μg/ml of rifampicin. Efficiency of plating (eop) is defined as the number of colonies obtained on complete-agar plates containing 10 μg/ml of rifampicin and incubated at 28 °C for 6 days, as a percentage of the colonies obtained on rifampicin-free complete-agar.

(v) *Determination of the minimal inhibitory concentration (MIC)*

With sonicated inoculum: a 3-day-old culture shaken in TS medium at 37 °C was sonicated for 1 min to form bacilli-like fragments; then complete-agar slants, containing the test antibiotic at a range of concentrations (increasing in 2-fold steps), were inoculated with it. Inoculum concentration was 10^7–10^8 colony forming units/ml. Inoculation was at 37 °C for 4 days.

With filtered inoculum: a culture grown on complete-agar slants for 6 days at 37 °C was washed off with 1 % Tween 80, vortexed and filtered using sintered G4 filters. 2–5·10^2 colony forming units were plated on complete-agar plates containing the test antibiotic at a range of concentrations increasing in 2-fold steps, and incubated at 37 °C for 6 days.

(vi) *RNA synthesis in osmotically shocked cells*

This method was based upon that of Gros *et al.* (1967).

Cells were grown in CGT medium at 37 °C in liquid culture. On reaching mid-log phase the culture was centrifuged, washed twice with a salt mixture buffered with Tris (composition identical with CGT, except that it was devoid of glucose and casamino acids), and suspended in $\frac{1}{5}$ volume of the same mixture supplemented with 2M saccharose. After 10 minutes incubation at room temperature, the suspension was diluted 5-fold with a salt mixture buffered with Tris, centrifuged and washed twice. The cells were resuspended in one tenth of their original volume in CGT medium containing 5 mM CTP, ATP and GTP, then 2 μCi/ml[^{3}H]-UTP was added, together with 0·5 mM unlabelled UTP, and the mixture incubated at 30 °C. The incorporation of radiolabel into the fraction insoluble in cold TCA was assayed, following filtration through Whatman GFC 25 filters, using a Packard liquid scintillator.

Rifampicin and actinomycin D were dissolved in ethanol, to give a final ethanol concentration of 1 % (v/v) in the mixture. Following osmotic shock survival was 1–5 %.

3. RESULTS

From a *Micromonospora sp.* strain S1/109 a mutant (no. 457), resistant to 160 μg/ml rifampicin was isolated by plating on 10 μg/ml rifampicin. Table 1 gives the rifampicin and novobiocin sensitivities of the wild and mutant strains, and shows that the novobiocin sensitivity of the rifampicin-resistant strain had increased 4-fold (measured in the sonicated inoculum) or eightfold (measured in filtered inoculum). A further increase in rifampicin resistance (to MIC = 800 μg/ml), achieved by selecting derivatives of mutant 457 able to grow in the presence of higher concentrations of the drug, produced no further increase in novobiocin sensitivity. Streptomycin resistance and auxotrophic mutations had no effect on novobiocin sensitivity.

In order to confirm that rifampicin resistance was not caused by decreased

Table 1. *Novobiocin and rifampicin sensitivity of* Micromonospora *sp.*

Inoculum	MIC (μg/ml) Novobiocin	Rifampicin
S1/109 (wild)		
Sonicated	64	0·05–0·1
Filtered	64	0·05–0·1
Mutant 457 (*rif*^{r})		
Sonicated	16	320
Filtered	8	160

These data summarize the results of ten different experiments.

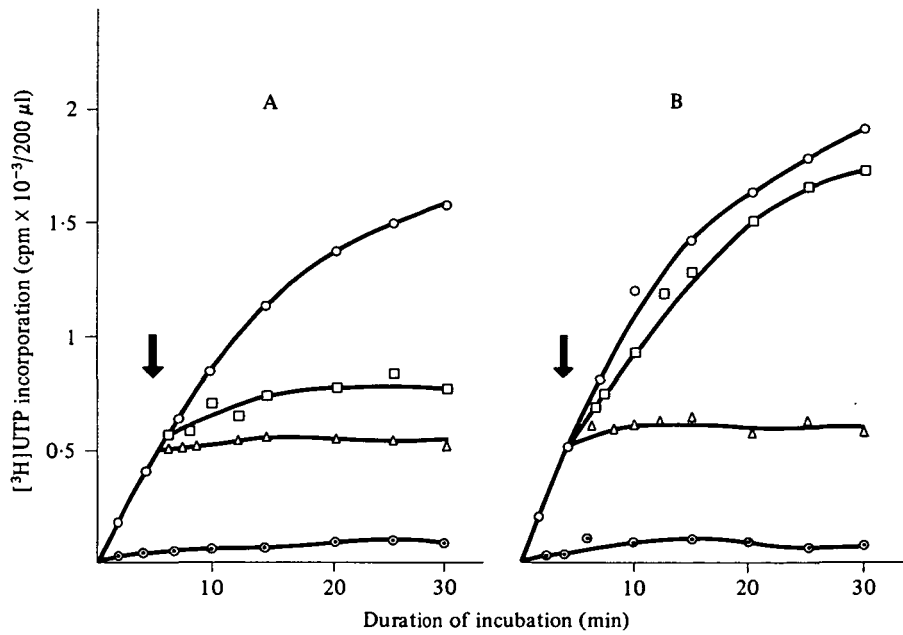

Fig. 1. The effect of rifampicin on [^{3}H]UTP incorporation in osmotically shocked cells of the wild (S1/109) strain (A) or *rif*^{r} (457) mutant (B). Antibiotic addition was in the 5th minute (arrow). Antibiotic free control (○), 20 μg/ml rifampicin (□), 5 μg/ml actinomycin D (△), ATP-free control (⊙).

permeability to rifampicin, but rather by a change in the DNA-dependent RNA polymerase (presumptive *rpoB* mutation), [^{3}H]UTP incorporation into the RNA of osmotically shocked cells was assayed in the presence of the other three nucleoside triphosphates (Fig. 1). With the rifampicin-sensitive strain 20 μg/ml rifampicin significantly inhibited triphosphate-dependent UTP incorporation, while with rifampicin resistant cells identical rifampicin levels had little effect. (With sensitive cells, even 1 μg/ml rifampicin caused inhibition, while with resistant cells even 50 μg/ml was ineffective). The incorporation of labelled UTP was

completely blocked by 5 μg/ml actinomycin D, confirming that the incorporation measured was due to the activity of DNA-dependent RNA polymerase. In this system 100 μg/ml novobiocin had no effect on RNA synthesis. During the test period a slight reduction of rifampicin concentration was observed by a biological assay (using intact mycelia). However, the rate of reduction was no higher in the resistant than in the sensitive strain; consequently degradation of the drug cannot be held responsible for rifampicin resistance.

Table 2. *Distribution of the novobiocin sensitivity of spontaneously* rif^r Micromonospora *sp. strains*

Mutant group	Novobiocin MIC (μg/ml)		
	16	32	64
1	21	4	0
2	30	0	0
3	8	2	0
4	2	0	2
5	0	0	4
6	10	0	0
7	2	1	2
8	5	0	0

Each group of mutants was derived from *rif*^{r} selection applied to a separately cultured single colony. The MIC was assayed with sonicated inoculum. MIC for the wild type was 64 μg/ml. Each strain was assayed 3 times.

Rifampicin-sensitive progeny, obtained as a few spontaneous back mutants without selection, exhibited the original novobiocin resistance.

The incidence of pleiotropy was then studied. In Table 2 the novobiocin sensitivity of 93 *rif*^{r} strains, isolated from cultures originating from 8 individual colonies, is summarized. A high incidence of novobiocin-sensitive strains is apparent. Generally, *rif*^{r} strains which failed to show pleiotropy (novobiocin MIC = 64 μg/ml) exhibited an eop lower than 100% (1–20%) on rifampicin-containing plates. Only two *rif*^{r} strains (both from group 7) showed a 100% eop on rifampicin, while remaining wild-type in their sensitivity to novobiocin. Slight differences in the proliferation rate of the *rif*^{r} mutants failed to show any correlation with novobiocin sensitivity.

The pleiotropic phenomenon could not be reproduced in a gentamicin-producing *Micromonospora echinospora* strain. The novobiocin sensitivity of this wild strain was already comparable to that of the *rif*^{r} mutant 457 of strain S1/109, the MIC value being 8 μg/ml assayed either with filtered or with sonicated inoculum. There was only a slight (2-fold) increase in the novobiocin sensitivity of spontaneous resistant mutants exhibiting 100% eop on rifampicin-containing plates (MIC = 4 μg/ml).

The specificity of the phenomenon was investigated by testing various antibacterial agents using sonicated and filtered inocula. The results obtained with

coumermycin A_1 (MIC of the wild strain = 0·5 μg/ml) were interesting: rifampicin resistance failed to induce an increase in sensitivity (there was even a slight, 2-fold, reduction). This is surprising since both the chemical structure, and the mode of action of coumermycin in *E. coli* are closely related to those of novobiocin. For the other compounds (data not shown) sensitivity either remained unchanged (mitomycin C, acridine orange, actinomycin D, mithramycin, streptomycin, neomycin, D-cycloserine, bacitracin, penicillin G) or was increased by about 2-fold (paromomycin, viomycin, erythromycin, chloramphenicol, oxytetracycline).

The effect of a combination of coumermycin A_1 and novobiocin was studied in wild and *rif*^{r} strains. In the *rif*^{r} mutant 457 the MIC value of novobiocin was enhanced by substatic levels of coumermycin A_1 (2-fold by 0·06–0·12, 4-fold by 0·25–0·5 μg/ml): thus, surprisingly, coumermycin protected the strain against novobiocin. The MIC value of coumermycin A_1 could not be increased by any substatic novobiocin concentration. The high novobiocin MIC value of the wild strain, on the other hand, could not be enhanced further by 0·06–0·25 μg/ml coumermycin A_1.

All strains tested were resistant to nalidixic acid (MIC > 200 μg/ml).

4. DISCUSSION

The above experiments show that we have detected a novel pleiotropic effect of a rifampicin resistance mutation (which, according to the experiment summarized in Fig. 1., is very probably an *rpoB* mutation of RNA polymerase). Both the complete correlation of novobiocin sensitivity and rifampicin resistance in the case of back mutation*, and the high incidence of spontaneous Rif-R Nov-S phenotypes reveal this phenomenon, since the simultaneous appearance of two independent spontaneous mutations is improbable.

In considering the mechanism of novobiocin-coumermycin antagonism it has to be assumed that coumermycin is not competing for the same site as novobiocin, as in this case the protective effect would be competitive. On the contrary, only a 2-fold decrease in novobiocin sensitivity was produced by an 8-fold increase in coumermycin concentration. We suggest that an allosteric effect, exerted at another site, may be responsible for the increase in the MIC value of novobiocin, in the presence of subtoxic levels of coumermycin. A similar novobiocin-coumermycin antagonism was observed with *Bacillus subtilis* strain 168 (unpublished data).

The mechanism of pleiotropy may be interpreted in the following alternative ways. (1) Novobiocin penetration is increased in the *rif*^{r} mutant, probably due to the impaired transcription of some membrane proteins participating in novobiocin exclusion. (2) Transcription in the *rif*^{r} mutant is more sensitive than normal to changes in the degree of supercoiling of the DNA.

The first of these hypotheses is implausible. If it were correct, the *internal* concentration of novobiocin required to block cell growth (via gyrase-inhibition)

* Selection of mutant 457 with novobiocin increased the probability of the occurrence of spontaneous *rif*^{s} back mutants (data not shown).

would be identical in the two strains. Our results indicate that penetration of coumermycin into the cells is, if anything, more efficient in the wild type strain. Thus the antagonistic effect of coumermycin against novobiocin-inhibition of growth should be as strong (or stronger) in the wild-type as in the *rif*^{r} mutant. In fact, however, the antagonism is detectable only in the *rif*^{r} strain.

For this reason, we favour hypothesis (2). The different MIC values of the two strains suggests that there is at bacteriostasis a different concentration of novobiocin at the surface of DNA gyrase, $\frac{1}{4}$–$\frac{1}{8}$ as much in the *rif*^{r} strain as in wild type. This is however sufficient to block the function of the mutant RNA polymerase, because it is more sensitive to partial uncoiling of DNA. Lower novobiocin concentrations may be antagonized by coumermycin as discussed earlier; but in the wild strain where higher levels of novobiocin are required to inhibit cell proliferation, this antagonism is not detectable.

This interpretation of pleiotropy is in good agreement with published data. In intact cells the novobiocin-coumermycin sensitivity of transcription (Smith & Davis, 1965; Michaeli *et al.* 1971; Ryan, 1976) has been explained by the requirement for supercoiled DNA in transcription (Botchan, Wang & Echols, 1973; Wang, 1974). According to Smith, Kubo & Imamoto (1978); Yang *et al.* (1979); and Sanzey (1979) the inhibition of transcription by gyrase inhibitors is promoter-specific. The preferential inhibition of rRNA synthesis is described by Oostra, Geert & Gruber (1980). Recently Mirkin *et al.* (1979) observed changes in coumermycin sensitivity produced by *rpoB* and *rpoC* mutations in *Escherichia coli*, and concluded that the dependence of RNA synthesis on the degree of DNA supercoiling can be altered by such mutations of RNA polymerase.

Sensitivity to coumermycin was not increased by the pleiotropic rifampicin resistance mutation in our organism, despite the finding that in *E. coli* the uncoiling effect of coumermycin is exerted through the same (B) subunit of DNA gyrase as that of novobiocin (Gellert *et al.* 1976). In view of the complex antagonism between coumermycin and novobiocin in *Micromonospora*, and the possible effect of *rif*^{r} mutation on permeability to coumermycin, it is not possible to interpret this seeming paradox without further study.

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