Genet. Res., Camb. (1984), **44**, *pp.* 243–250
With 5 text figures
Printed in Great Britain

Antagonism between novobiocin and coumermycin A_1 in *Bacillus subtilis*

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(*Received 5 December 1983 and in revised form 18 June 1984*)

SUMMARY

When combinations of inhibitors acting on the subunit B of DNA gyrase were tested in *Bac. subtilis* strains, the growth-inhibiting effect of novobiocin was specifically antagonized by subinhibitory concentrations of coumermycin A_1. An antagonism in the opposite direction was not observed.

Two alternative models are proposed, where the supercoiling decrease caused by novobiocin is antagonized by coumermycin.

This phenomenon seems to be characteristic of the *Bac. subtilis* species.

1. INTRODUCTION

The *Escherichia coli* DNA gyrase (Eco topo-isomerase II; (Gellert *et al.* 1976*a*) is an essential enzyme which is required for several processes involving DNA, e.g. replication, transcription and recombination (Cozzarelli, 1980; Gellert, 1981). It introduces negative supercoils into closed DNA duplexes using energy gained through ATP-hydrolysis (Sugino *et al.* 1978). This enzyme is a tetramer of 2-2 subunits A and B, coded by genes *gyrA* and *gyrB* (Higgins *et al.* 1978; Mizuuchi, O'Dea & Gellert, 1978; Hansen & von Meyenburg, 1979). Subunit A is the target of nalidixic acid and oxolinic acid (Sugino *et al.* 1977), while subunit B is that of nov and cou (Mizuuchi, O'Dea & Gellert, 1978).* These latter drugs competitively inhibit the binding of ATP to subunit B (Sugino *et al.* 1978). Nov and cou exhibit cross-resistance (Gellert *et al.* 1976*b*).

An enzyme analogous with respect to structure and function has been described in *Bacillus subtilis* 168 (Sugino & Bott, 1980; Orr & Staudenbauer, 1982). It is probable that gyrase-type topo-isomerases are present in all prokaryotes.

When antibacterial agents having identical modes of action are combined, additive effects can be predicted, but synergism or antagonism cannot be expected and their description in the literature is rare. As regards DNA gyrase inhibitors, synergism between nalidixic acid and nov was reported (Chao, 1978). Antagonism

* Abbreviations: nov, novobiocin; cou, coumermycin A_1; cfu, colony forming unit; MIC, minimal inhibition concentration.

has only been described between nov and chloramphenicol, erythromycin, or lincomycin (Garrett Won, 1973). Antagonism or synergism between DNA gyrase inhibitors acting on the same subunit has not been described.

In the present work antagonism is reported between nov and cou.

2. MATERIALS AND METHODS

(i) *Organisms*

The strains used are summarized in Table 1.

(ii) *Materials*

Novobiocin sodium salt was from Sigma, rifampicin and mitomycin C were from Serva, chloramphenicol was from EGA-Chem., penicillin G was from Biogal, nalidixic acid was from Chinoin. Coumermycin A_1 was a gift of J. Berger (Hoffman-LaRoche). It was dissolved in dimethylsulphoxide. In all experiments dimethylsulphoxide controls were performed, which were negative.

(iii) *Media*

Oxoid nutrient broth or nutrient agar was used in all experiments.

(iv) *Qualitative agar diffusion test*

Nutrient agar plates (16 ml petri dishes) were overlaid with 4 ml of the same agar inoculated with 10^6 cells/ml. Samples (0·1 ml) of different DNA gyrase inhibitor solutions were taken into the holes in alternating order. The plates were incubated at 37 °C overnight, then stained with iodine–nitro-tetrazolium chloride.

(v) *Quantitative agar diffusion test*

Nutrient agar plates were inoculated as in the qualitative test. Nov solutions (0·1 ml) were taken into the holes in doubling steps of concentration. Subinhibitory cou concentrations were mixed into both layers of the agar.

(vi) *Growth experiment*

It was carried out in tubes containing 5 ml medium, without shaking; incubation was at 37 °C. An overnight culture was used for inoculation. The optical densities were measured on a Spectromom 402 photometer at 620 nm; the numbers of colony forming units (cfu) were determined by plating on nutrient agar. All experiments were made in triplicate.

3. RESULTS

An antagonism between nov and cou was observed originally in a sisomicin-producing *Micromonospora* sp. (Gadó *et al.* 1982). This phenomenon has been studied in more detail in *B. subtilis* strain 168.

At first we carried out a qualitative agar-diffusion test. The antagonism was clearly visible (Fig. 1): the inhibition zones of nov were deformed by cou applied in a suitable concentration. An effect in the opposite direction could not be observed.

Table 1. *Bacterial strains used for experiments*

Species	Strain	Genotype or phenotype	Source
Bacillus subtilis	168	thymine-requiring	4
	BD 430(pE194^x)	trpC2 thr-5	2
	ATCC 6633		3
	ATCC 6051		3
	ATCC 9799		3
	NCTC 10073		3
	GSY 244	pheA1, ilvC1	4
	GSY 384	argA2, leu-1	4
	BD 11	purA16,leu-8,metB5,str-1, ery-1,mic-1	4
	BD 13	try-2,argC4,leu-2	4
	BD 34	thr-5,leu-8,metB5	4
	BD 46	purA16,leu-8,metB5,lys-21	4
	BD 59	argC4	4
	BD 71	hisA1,argC4,ura-1	4
	BD 99	thr-5,hisA1,try-2	4
	BD 115	aro-2,try-2,hisB2,try-1	4
	BC 369	hisA1,argC4,metD1,pha-1	4
	PG 594	trpC2,metC3,mtlB1	4
	SB 19E	tslA13,cdd-1,dck-3,crk-7	5
B. megaterium	ATCC 15374		3
	KM	Tryptophan, histidine, threonine-requiring	1
B. cereus	ATCC 10702		3
Staphylococcus aureus	484(pE194)*		2
Escherichia coli	AS-19 (permeability mutant)		1

* Macrolide–lincosamid–streptogramin B resistance is coded by plasmid pE194.

Sources of strains: (1) L. Alföldi, Biological Center of Hungarian Academy of Science at Szeged, Hungary; (2) L. Jánosi, National Institute of Health, Budapest; (3) National Collection of Microorganisms, Budapest; (4) J. Molnár, Medical School of Szeged; (5) I. Takahashi, McMaster University, Hamilton, Ontario, Canada.

In quantitative agar-diffusion assay the diameters of inhibition zones of nov were decreased by subinhibitory concentrations of cou mixed into the agar medium (Fig. 2).

The antagonism was also studied in growth experiments with *B. subtilis* strain 168, monitored by the determination of optical density and the count of colony forming units (cfu).. Cou applied two hours later than nov exhibited a protective effect similar to that seen if the drugs were added simultaneously, in respect of intensity and kinetics of growth (Fig. 3). The protective effect of subinhibitory cou concentrations was most significant in a definite nov concentration range (Fig. 4). The inhibitory effect of 0·75–3 μg/ml nov on the increase of cfu could be antagonized by 0·03–1 μg ml cou. Note that a 30-fold increase in cou concentration could only protect to an extent equivalent to a twofold increase in nov level. Some antagonism could also be observed at very low cou concentrations, e.g. 0·003 μg/ml; and it was also detectable as a decrease in the filament formation caused by nov.

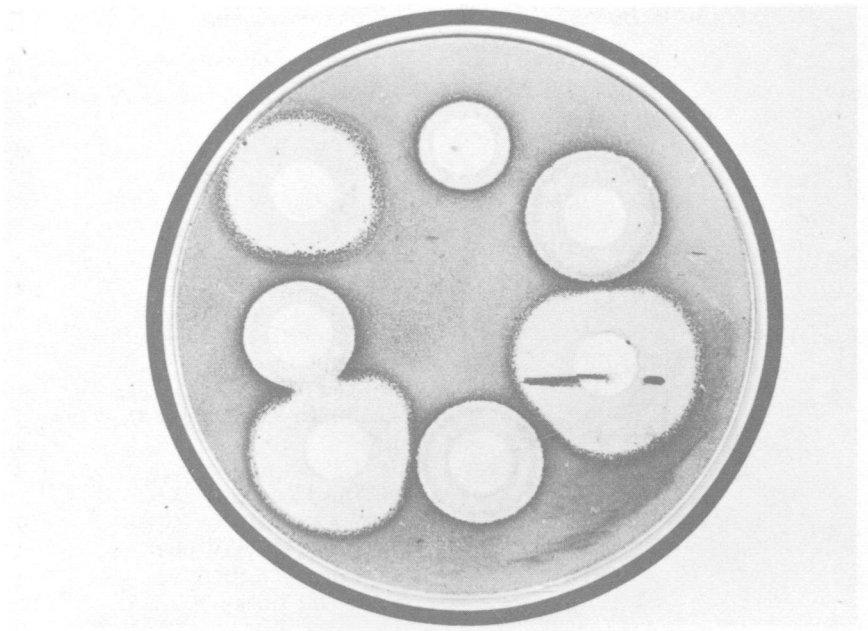

Fig. 1. Antagonistic effect of coumermycin A_1 against novobiocin in *Bacillus subtilis* 168: qualitative agar-diffusion test. Samples (0·1 ml) of DNA gyrase inhibitor solutions were measured into the holes beginning from the marked hole (clockwise): 25, 12·5 and 6·25 μg/ml nov (holes 1, 3 and 5); 12·5, 6·25, 3·12 and 25 μg/ml cou (holes 2, 4, 6 and 7).

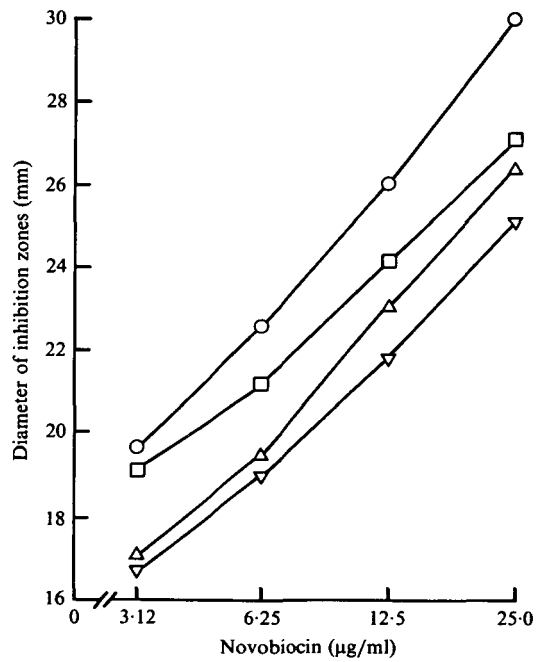

Fig. 2. Antagonistic effect of coumermycin A_1 against novobiocin in *Bacillus subtilis* 168: quantitative agar-diffusion test. Cou concentrations mixed into the medium: ○, control, 0·003 μg/ml; △, 0·03 μg/ml; ▽, 0·3 μg/ml.

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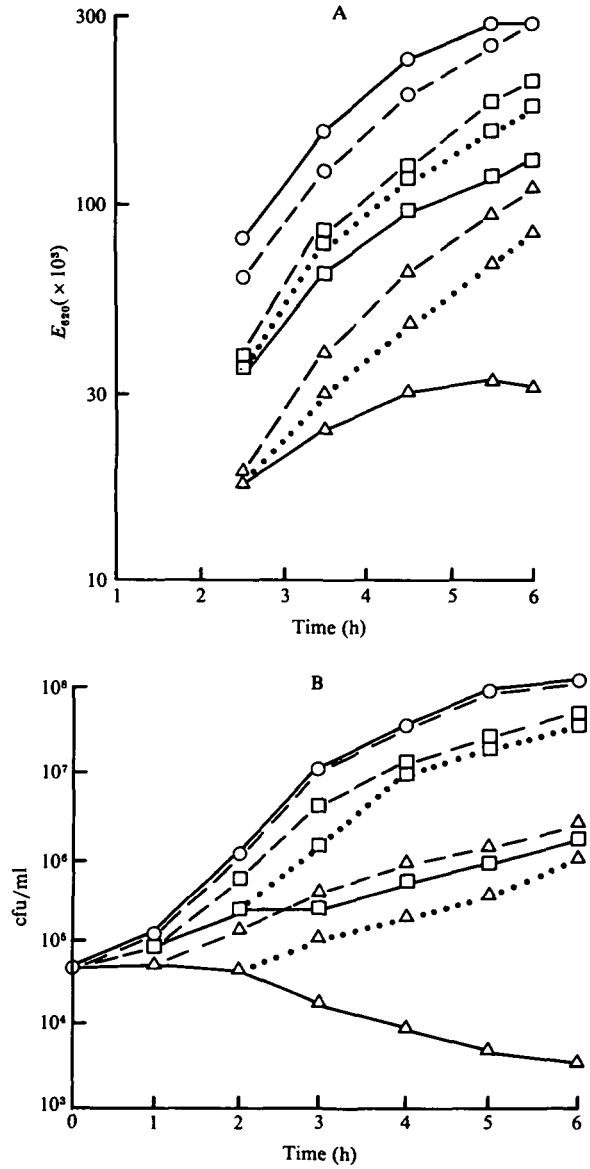

Fig. 3. Antagonistic effect of coumermycin A_1 against novobiocin in *Bacillus subtilis* 168: growth experiment. Growth was followed by optical density measurement (A) or by the count of cfu (B). Nov concentrations: ○, nov-free; □, 1 μg/ml; △, 2 μg/ml. Cou (0·3 μg/ml) was added simultaneously (broken lines) and 2 h later (dotted lines). Cou-free controls are marked with solid lines.

On the other hand, subinhibitory nov concentrations failed to reduce the inhibitory effect of cou in the growth experiments.

In the case of some other antibiotics (mitomycin C, rifampicin, chloramphenicol, penicillin G and nalidixic acid) no protective effect of cou could be observed in growth experiments (data not shown). Qualitative agar-diffusion tests excluded any interaction between nalidixic acid and cou, or nalidixic acid and nov.

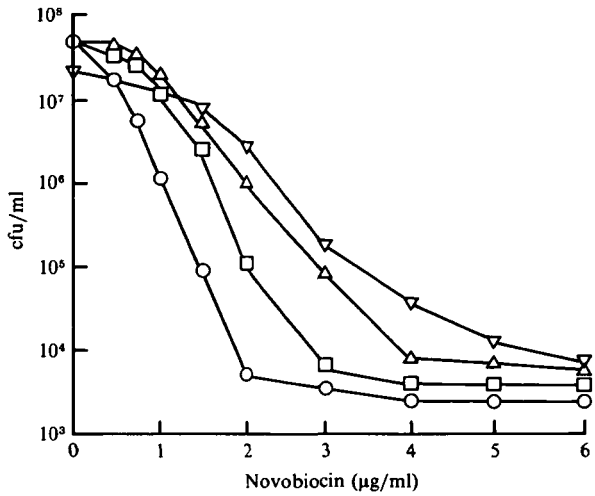

Fig. 4. Antagonistic effect of various coumermycin A_1 concentrations against novobiocin in *Bacillus subtilis* 168: growth experiment. Growth was followed with the count of cfu. Incubation time: 6 h. Inoculum: $5 \cdot 10^4$ cfu/ml. MIC of cou for the strain tested is 4 μg/ml. Cou concentrations: ○, control; □, 0·03 μg/ml; △, 0·3 μg/ml; ▽, 1 μg/ml.

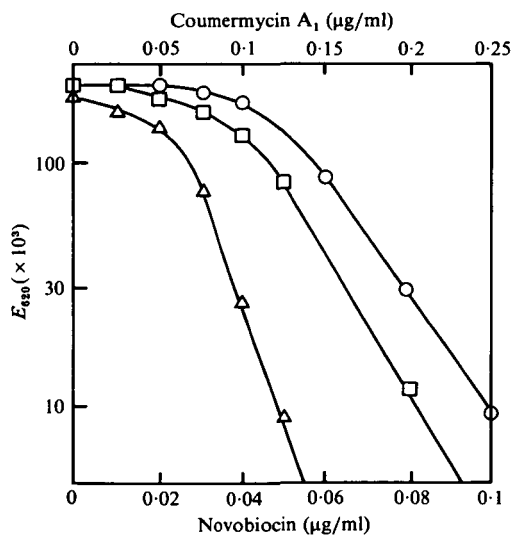

Fig. 5. Synergism between novobiocin and coumermycin A_1 in *Escherichia coli* AS-19: growth experiment. Growth was followed by optical density measurement. ○, Cou control; □, nov control; △, nov + 0·05 μg/ml cou.

We studied whether this antagonism is a general feature of *B. subtilis* species. Nineteen strains collected from various sources (Table 1) were screened using the qualitative agar diffusion test. All but one strain exhibited some deformation by cou of the inhibition zones caused by nov. Strain ATCC 6633 gave a negative result; however, quantitative agar-diffusion tests and growth experiments showed the

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usual cou–nov antagonism. Antagonism in the opposite direction was excluded by the qualitative test in all strains.

Two *B. megaterium* strains were examined by all three methods. Neither showed antagonism, in either direction.

In the case of *B. cereus* ATCC 10702 cou did not antagonize nov in the diffusion tests. A slight protective effect of nov against cou, of uncertain significance, was found only in growth experiments. (Data not shown.)

Staphylococcus aureus 484 (pE194) showed no antagonism by any test, in either direction.

In an *E. coli* mutant (strain AS-19), which unlike the wild strain is nov sensitive, nov and cou exhibited synergism in growth experiments (Fig. 5).

4. DISCUSSION

The protective effect of cou against growth inhibition caused by nov may be explained in five different ways.

(1) Antagonism at a metabolic level. According to the literature the point of action of nov and cou is exclusively the subunit B of DNA gyrase.* Our experiments carried out with some other antibiotics suggest than nov is antagonized in a specific way. Thus it seems improbable that cou would antagonize the metabolic consequences of partial gyrase inhibition.

(2) One may propose a partial inhibition of nov penetration into the cell by cou. Although this possibility could not be excluded, the results of the experiment in which cou was applied later than nov make this unlikely.

(3) (*a*) It could be assumed that a competition takes place between nov and cou for the same site on the subunit B of DNA gyrase. It is known, however, that both drugs inhibit DNA gyrase by exlcuding ATP from a common target, thus at its effective concentration cou would also disturb the binding of ATP and would not antagonize the growth inhibition.

(*b*) Cou is bound to an additional site in the subunit B, where it interferes allosterically with the binding of nov on another site of the same subunit, thus enhancing the possibility of ATP binding. This effect is not in strict correlation with the cou concentration: a 30-fold increase in cou dose produced protection corresponding to only a twofold increase in nov dose (Fig. 4). Nov has no such effect.

(*c*) Subinhibitory cou concentrations inhibit a topoisomerase having relaxing activity (e.g. topo-isomerase II′). In this way it increases the negative supercoiling of DNA reduced by nov. Cou added alone in these doses inhibits equally both enzymes, thus the level of supercoiling remains undisturbed. In higher doses there is an overwhelming inhibition of gyrase. Nov does not act on the relaxing enzyme.

Our results do not make it possible to decide between models 3*b* and 3*c*.

Preliminary experiments have indicated that cou can antagonize the plasmid curing effect of nov in *B. Subtilis* Bd 430 (pE194) (unpublished data).

Contrary to the literature (Cozzarelli, 1980; Gellert, 1981) our results suggest that the effects of nov and cou exhibit a qualitative difference. Further, there may

* Cou inhibits DNA polymerase II and RNA polymerase of *E. coli* (Ryan & Wells, 1976) *in vitro*, when applied in high concentration. This fact has no likely biological significance.

be differences not only between the DNA gyrases of *E. coli* and *B. subtilis*, but also among taxonomically more related species. The 19 *B. subtilis* strains uniformly exhibited the unidirectional cou–nov antagonism, which seems to be a stable character of this species.

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