Inhibition of Sporulation by DNA Gyrase Inhibitors

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The effects of oxolinic acid and novobiocin – which respectively inhibit the A and B subunits of DNA gyrase, and therefore inhibit DNA synthesis – have been examined in sporulating cultures of Bacillus subtilis. Although both inhibitors prevent sporulation, this is not due to inhibition of DNA synthesis. Instead, they affect protein synthesis generally, though weakly, but have very marked effects on the formation of individual enzymes. These effects are reproducible, but the type of enzyme that will be affected is not predictable. The results point to an involvement of DNA gyrase in the transcription of some genes. This is suggested as the reason for the effect of the inhibitors on spore formation, which they block mainly at Stage O–I.

INTRODUCTION

The experiments of Mandelstam et al. (1971) established a relationship between DNA replication and induction of sporulation in Bacillus subtilis. They showed that thymine deprivation in thymine auxotrophs resulted in inhibition of sporulation and of the associated biochemical events such as production of extracellular proteases, ribonuclease and alkaline phosphatase. Later, Dunn et al. (1978) showed that 6-(p-hydroxyphenylazo)uracil (HPUra), a specific inhibitor of DNA polymerase III (Gass et al., 1973) stopped sporulation if the inhibitor was added at the time when sporulation was induced, and that the cell population escaped progressively from the effect of the inhibitor as the cells completed chromosome replication.

Other inhibitors have recently been shown to have a preferential effect on DNA synthesis, e.g. novobiocin, oxolinic acid and nalidixic acid. All these affect the action of the enzyme DNA gyrase (Gellert et al., 1977), which controls the extent of supercoiling of DNA and which is necessary for DNA replication (Sugino et al., 1977; Gellert et al., 1977). Gyrases have been isolated from Escherichia coli (Gellert et al., 1976), Micrococcus luteus (Liu & Wang, 1978) and B. subtilis (Sugino & Bott, 1980) and shown to have similar properties.

In this paper the effects of DNA gyrase inhibitors on sporulation are compared with those produced by HPUra. The results show that inhibition of sporulation by gyrase inhibitors cannot be accounted for by their effect on DNA synthesis and is more likely to result from an effect on RNA synthesis.

METHODS

Organisms. Bacillus subtilis Nil, a derivative of strain 168trpC2, was used in most of the experiments. It is auxotrophic for thymine and tryptophan and carries a defective PBSX phage in its genome which does not induce lysis of the cells (Karamata, 1968). Bacillus subtilis 168 trpC2 (Marburg) was used for electron microscopy experiments because it sporulates better than strain Nil. It requires tryptophan for growth.

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Growth of cells and resuspension in sporulation medium. The organisms were grown in casein hydrolysate (CH) medium to a density of about 0·25 mg dry wt ml⁻¹. Cultures were centrifuged and the cells resuspended to the same density in sporulation medium (Sterlini & Mandelstam, 1969). Times (h) after resuspension are denoted t₁, t₂, etc.: sporulation, which is not as good in strain Nil as in the wild-type, was usually maximal (about 40% incidence) by t₄. Growth and sporulation were carried out at 37 °C with vigorous aeration. Bacterial growth was measured spectrophotometrically using a calibration curve relating turbidity at 600 nm to bacterial dry weight.

Spore incidence was measured by counting refractile spores under a phase-contrast microscope. At least 400 cells were counted for every determination.

Protease. Serine-protease was measured as described by Dancer & Mandelstam (1975). A unit of protease is defined as the activity which solubilizes 1 mg Remazol Brilliant Blue-Hide in 30 min at 37 °C.

Alkaline phosphatase. This enzyme was assayed by the method of Glenn & Mandelstam (1971). One enzyme unit liberates 1 nmol p-nitrophenol min⁻¹ at 37 °C.

Amylase. Amylase was assayed by a modification of the method described by Radley (1943). To samples of supernatant (1 ml) at 37 °C, soluble starch (0·5 ml, 0·25% w/v) in phosphate buffer pH 7·0 was added. After 30 min, the reactions were stopped by adding HCl (0·5 ml, 1 M). Iodine (0·1%, w/v) in KI (1%, w/v) was added (0·1 ml) and the absorbance of the solution was measured at 680 nm against a blank in which HCl was added before the soluble starch. The readings for the samples were subtracted from that for the blank and the resulting values are expressed in arbitrary units.

Histidase. The enzyme was measured as described by Coote (1974). One unit is defined as the amount which produces 1 nmol urocanic acid min⁻¹ at 30 °C.

DNA synthesis. This was followed by measuring incorporation of [methyl-3H]thymidine into DNA. Cells growing in CH medium were resuspended in sporulation medium containing [methyl-3H]thymidine (0·2 µCi ml⁻¹) and carrier thymidine (20 µg ml⁻¹). Samples (1 ml) were taken at intervals and fixed in trichloroacetic acid (TCA) (1 ml, 10%, w/v). The samples were filtered by suction through glass-fibre filters (Whatman GF/C, 2·1 cm diam., 1·2 µm pore size). The filters were washed with TCA (10 ml; 5%, w/v) containing thymidine (300 µg ml⁻¹) and then with ethanol (5–10 ml; 96%, v/v). They were dried in a vacuum oven at 80 °C for 60 min and transferred to plastic scintillation vials containing 5 ml scintillation fluid [2·4(4'-tert-butylyphenyl)-5(4'-biphenylyl)-1,3-oxadiazole (butyl-PBD); 0·5%, w/v, in toluene]. Radioactivity was counted in a Wallac LKB 1215 Rack Beta scintillation counter using the module for 3H.

Protein synthesis. This was followed in a similar manner to DNA synthesis. [15N]Methionine (0·2 µCi ml⁻¹) in carrier methionine (20 µg ml⁻¹) was used instead of [methyl-3H]thymidine. Radioactivity in the TCA-insoluble fraction was measured by using the 14C module.

Electron microscopy. Cells were fixed with osmium tetroxide, stained with uranyl acetate, dehydrated with ethanol and embedded in Araldite. Sections were cut, stained with lead citrate and examined using a Philips 200 electron microscope (see Kay & Warren, 1968).

Inhibitors. 6-(p-Hydroxyphenylazo)uracil (HPUra) and oxolinic acid were a gift from B. Langley of Imperial Chemical Industries. Novobiocin was purchased from Sigma and nalidixic acid from Calbiochem.

RESULTS

Inhibition of sporulation by DNA gyrase inhibitors

The concentrations required to reduce sporulation to less than 0·25% were determined for novobiocin and oxolinic acid. Cells resuspended in sporulation medium were incubated in the presence of graded concentrations of each inhibitor. Virtually complete inhibition of sporulation was obtained with oxolinic acid at 100 µg ml⁻¹ and novobiocin at 10 µg ml⁻¹. However, nalidixic acid, even at concentrations as high as 250 µg ml⁻¹, did not reduce the incidence of spores by more than 65%. Similar results with nalidixic acid were obtained by Keynan et al. (1976).

Effect of DNA gyrase inhibitors on DNA synthesis

The first possibility considered was that oxolinic acid and novobiocin were preventing sporulation by inhibiting DNA synthesis. Accordingly the effects of these inhibitors on DNA synthesis were measured at the concentrations established in the preliminary experiments. Strain Nil was grown in CH medium and resuspended in sporulation medium containing [methyl-3H]thymidine. The effects of the gyrase inhibitors and of HPUra on synthesis of DNA were determined in portions of the culture.
Inhibition of sporulation

Fig. 1. Effect of DNA gyrase inhibitors and HPUra on DNA synthesis by cells in sporulation medium. Strain NiI was grown in CH medium (125 ml) and resuspended in sporulation medium (125 ml) containing [methyl-3H]thymidine (0.2 μCi ml⁻¹) and carrier thymidine (20 μg ml⁻¹). The culture was split into five equal portions with the following additions: none (control) (○); nalidixic acid (250 μg ml⁻¹) (●); oxolinic acid (100 μg ml⁻¹) (△); novobiocin (10 μg ml⁻¹) (▲); HPUra (50 μg ml⁻¹) (□). DNA synthesis was followed as described in Methods. Samples were taken at intervals. The incidence of spores, expressed as a percentage at t₁₅₀, is shown for each culture.

HPUra, which inhibits DNA synthesis specifically, i.e. by blocking DNA polymerase III (Gass et al., 1973), completely prevented both sporulation and DNA synthesis (Fig. 1). The gyrase inhibitors at the concentrations tested did not stop DNA synthesis, although the onset of synthesis was delayed in cells treated with nalidixic acid or novobiocin. However, the amount of DNA finally synthesized in these cultures was about the same as in the control. Oxolinic acid delayed the onset of synthesis and also reduced the rate, so that even by t₄ the amount of DNA synthesized was only about 60% of that in the control (Fig. 1).

Escape of sporulation from the effects of DNA gyrase inhibitors

The experiments described above showed that concentrations of oxolinic acid and novobiocin that were inhibitory for sporulation still allowed a considerable amount of DNA synthesis to occur. Nevertheless, it was possible that chromosome completion had not occurred in the inhibited cultures. To check this possibility, the escape times for sporulation were determined with these inhibitors. The experiments were similar to those of Dunn et al. (1978) who showed that escape from HPUra begins about 35 min after resuspension and coincides with the time at which the first cells to be initiated to sporulation complete their chromosomes. Nalidixic acid was not used in this or the subsequent experiments because of its failure to stop either sporulation or DNA synthesis.

Strain NiI was grown in CH medium and resuspended in sporulation medium. Portions of the culture were taken at intervals starting from t₀, treated with inhibitor, and incubated to t₁₅₀, and the incidence of refractile spores was measured. The escape time for HPUra-treated cells was about 35 min (Fig. 2), similar to that reported by Dunn et al. (1978). With oxolinic acid, a significant number of spores appeared in cultures to which the inhibitor was added only 15 min after resuspension, whereas with novobiocin the escape occurred much later, 75–90 min after resuspension (Fig. 2).
Incubation time (min)

Fig. 2. Times of escape of sporulation from the effects of DNA gyrase inhibitors and of HPUra. Strain Nil was grown in CH medium (150 ml) and resuspended in sporulation medium. Portions (10 ml) of the culture were withdrawn at intervals and treated with inhibitor: oxolinic acid (100 µg ml⁻¹) (○); HPUra (50 µg ml⁻¹) (△); novobiocin (10 µg ml⁻¹) (□). The incidence of refractile spores was scored at t₁₈.

Effect of DNA gyrase inhibitors on early biochemical events associated with sporulation

Since inhibition of sporulation by gyrase inhibitors was not correlated with inhibition of DNA synthesis, a relationship was sought between inhibition of sporulation and the effect of the inhibitors on production of some sporulation-associated enzymes.

Cells of strain Nil were grown in CH medium and then resuspended in sporulation medium containing either oxolinic acid or novobiocin. The effects of the inhibitors on the production of sporulation-associated enzymes – serine-protease and alkaline phosphatase – and of ‘vegetative’ enzymes such as α-methylase and the inducible enzyme histidase were followed and compared with those produced in cells treated with HPUra.

Serine-protease. Neither the gyrase inhibitors nor HPUra stopped the production of serine-protease, and the enzymic activity in the presence of the inhibitors varied from 60 to 80% of the maximum found in the control (Fig. 3a).

Alkaline phosphatase. Production of the enzyme was affected to a different extent by the inhibitors: oxolinic acid and HPUra reduced the enzymic activity by about half, while novobiocin was more inhibitory (Fig. 3b).

Amylase. The production of amylase, a ‘vegetative’ enzyme, was not significantly decreased by any of the gyrase inhibitors or by HPUra (result not shown).

Histidase. This enzyme was fully produced by cells treated with HPUra, and only slightly reduced by oxolinic acid (Fig. 3c). By contrast, novobiocin markedly inhibited production of the enzyme.

Effect of oxolinic acid, novobiocin and HPUra on protein synthesis during sporulation

The differing effects of the inhibitors on enzyme synthesis raised the possibility that inhibition of sporulation by the gyrase inhibitors might reflect their action on protein synthesis generally. Accordingly, Nil cells were grown in CH medium and resuspended in sporulation medium containing [³⁵S]methionine to measure protein synthesis. Novobiocin, oxolinic acid or HPUra were added at t₀ and at the approximate escape time for each inhibitor, i.e. 40 min and 90 min after resuspension.

In the cultures treated at t₀, HPUra and oxolinic acid had little effect for the first 30 min. There was then a progressively increasing inhibition, especially in oxolinic acid-treated cells (Fig. 4a). In the cultures treated at 40 min, the inhibitory effect of oxolinic acid had largely disappeared, while the effect of HPUra remained virtually unchanged (Fig. 4b).
Inhibition of sporulation

Fig. 3. Effect of DNA gyrase inhibitors on production of serine-protease (a), alkaline phosphatase (b) and inducible histidase (c). Strain NlI was grown in CH medium (250 ml) and the cells were resuspended in sporulation medium (250 ml). The solution also contained L-histidine (500 µg ml⁻¹), to induce histidase at the same time. The culture was split into four equal portions and received: no inhibitors (control) (○); oxolinic acid (100 µg ml⁻¹) (●); novobiocin (10 µg ml⁻¹) (△); HPUrA (50 µg ml⁻¹) (▲). Samples were taken at intervals for measurement of the three enzyme activities. The incidence of spores, expressed as a percentage at t₁₀, is shown for each culture in (a).

Fig. 4. Effects of oxolinic acid, novobiocin or HPUrA on protein synthesis during sporulation. Strain NlI was grown in CH medium (200 ml) and resuspended in sporulation medium. This culture was split into nine portions. (a) At t₀, three of the subcultures received either HPUrA (50 µg ml⁻¹) (●), or oxolinic acid (100 µg ml⁻¹) (△), or novobiocin (10 µg ml⁻¹) (▲); a fourth was kept as control (○). (b) At t₄₀min, two subcultures received HPUrA (●) or oxolinic acid (△) and a third was kept as control (○). (c) At t₉₀min, one subculture received novobiocin (▲) and another was kept as control (○). Each culture was treated with [³⁵S]methionine (0.2 µCi ml⁻¹) and carrier L-methionine (20 µg ml⁻¹) at the time of addition of inhibitor. Protein synthesis was followed for 100 min periods, as described in Methods.
Novobiocin at $t_0$ had a much more marked effect on protein synthesis than either of the other inhibitors; but in the 90 min cultures this, too, had largely disappeared (compare Figs 4 a and 4 c).

**Morphology of cells in sporulation medium treated with oxolinic acid or novobiocin**

The stage at which novobiocin or oxolinic acid inhibit the sporulation process was also investigated by electron microscopy. *Bacillus subtilis* 168 was used in this experiment. Samples for electron microscopy were taken at $t_0$.

Multiple septation was observed in nearly 20% of the cells treated with novobiocin; some of the septa appeared very thick, suggesting the presence of cell wall. These cells resembled some of the stage II mutants described by Waites *et al.* (1970).

About 90% of the cells treated with oxolinic acid had the appearance of normal vegetative cells, i.e. they were blocked at stage O-I of sporulation, although about 10% of them were elongated and contained several mesosomes, especially in polar areas (result not shown).

**DISCUSSION**

The effect of DNA gyrase inhibitors on sporulating *B. subtilis* cells has been followed and the results have been compared with those obtained with HPUrA, a specific inhibitor of DNA polymerase III.

The rapid and complete inhibition of DNA synthesis by gyrase inhibitors observed in *E. coli* (Goss *et al.*, 1965; Smith & Davis, 1967; Standenbauer, 1976) and by us in exponentially growing cells of *B. subtilis* (unpublished results) is not observed in cells in sporulation medium. In the latter medium, DNA synthesis is not stopped but only delayed by the inhibitors.

The inhibition of sporulation by gyrase inhibitors is thus not correlated with prevention of DNA synthesis. If it had been, the escape times measured with oxolinic acid and novobiocin would have been the same as that measured with HPUrA and would have corresponded with the completion time of chromosome replication (Dunn *et al.*, 1978).

The evidence presented in this paper suggests instead that inhibition of sporulation results from an effect of gyrase inhibitors on protein synthesis. It is clear that oxolinic acid and, more markedly, novobiocin greatly reduce the rate of protein synthesis, as measured by incorporation of methionine, when added at the onset of sporulation. This effect has become attenuated by the time the cells have reached the approximate escape time for the inhibitor concerned. However, the effects of the inhibitors on individual enzymes are erratic. Thus, novobiocin greatly reduces production of alkaline phosphatase and of histidase, has much less effect on protease and none on amylase, while oxolinic acid has a severe inhibitory effect only on alkaline phosphatase.

At the concentrations used, both novobiocin and oxolinic acid prevent the appearance of refractile spores and most of the cells are blocked somewhere between stage O and stage II of sporulation.

The preferential inhibition of synthesis of some enzymes suggests that DNA gyrase activity is necessary for the transcription of some genes but not others. Indeed, evidence has been adduced from experiments in *E. coli* that transcription is affected by these inhibitors (Puga & Tessmann, 1973; Ryan, 1976; Smith *et al.*, 1978; Sanzey, 1979). It has also been shown that novobiocin inhibits spore outgrowth in *B. subtilis*, a process which is independent of DNA synthesis (Ginsberg & Keynan, 1978; Gottfried *et al.*, 1979). This again suggests an effect on transcription.

The fact that the two gyrase inhibitors affect the synthesis of different enzymes differently, together with the fact that sporulation escapes from their inhibitory effects at widely separated times, suggests the possibility that the A and B subunits of DNA gyrase – the first being inhibited by oxolinic acid and the second by novobiocin – play separate roles, so that a
gene whose transcription is seriously decreased by one inhibitor may be insensitive to the other.

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