Nitrofurantoin Prompts the Stringent Response in *Bacillus subtilis*

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(Received 13 January 1981; revised 24 April 1981)

Nitrofurantoin causes the stringent response in *Bacillus subtilis*. After exposure of a stringent strain to this drug, the intracellular concentrations of guanosine 3'-diphosphate 5'-diphosphate (ppGpp), guanosine 3'-diphosphate 5'-triphosphate (pppGpp) and ATP increased, while that of GTP decreased. In a relaxed strain no accumulation of ppGpp or pppGpp was observed, but both GTP and ATP declined after the addition of nitrofurantoin. Protein synthesis was equally sensitive to nitrofurantoin in both the stringent and relaxed strains, but the drug inhibited RNA accumulation only in the stringent strain, not in the relaxed strain. Nitrofurantoin also caused the accumulation of ppGpp in *Escherichia coli* and *Serratia marcescens*.

INTRODUCTION

Nitrofurantoin, i.e. 1-[(5-nitrofurfurylidene)amino]hydantoin, is a nitrofuran derivative with broad antimicrobial activity which is widely used in therapy and as a general antimicrobial agent (for review, see Grunberg & Titsworth, 1973; McCalla, 1979). It is, however, still not known with certainty which target in bacterial metabolism is mainly responsible for its antimicrobial effect. Nitrofurans have been reported to interfere with carbohydrate metabolism (Hamilton-Miller & Brumfitt, 1976), with the synthesis of RNA and polysome assembly (Tu & McCalla, 1976), and with the translation of the mRNA of enzymes subjected to catabolite repression (Herrlich & Schweiger, 1976). The mutagenic and carcinogenic properties of nitrofurans are well established (Anon., 1976; McCalla, 1979).

We were interested in studying the mode of action of nitrofurantoin in *Bacillus subtilis*, since in this organism nitrofurantoin initiates sporulation in rich media in which sporulation usually occurs only at a very low frequency (Fortnagel, 1981). Sporulation of *B. subtilis* in rich media can be initiated by inhibition of purine synthesis, for example by the addition of inhibitors of purine synthesis (Mitani *et al.*, 1977; Heinze *et al.*, 1978), or by using strains blocked in the purine pathway (Freese *et al.*, 1979). In all these cases a decrease in the intracellular concentrations of GTP and GDP (but not necessarily of ATP) accompanied the initiation of sporulation. The relief of the shortage of guanine nucleotides by addition of guanine or guanosine inhibited sporulation (Lopez *et al.*, 1979). Deprivation of carbon or nitrogen sources, which promotes sporulation, also caused a decrease in the guanine nucleotide pools (Lopez *et al.*, 1981). In stringent strains, this decrease is caused by ppGpp, which inhibits the formation of xanthosine monophosphate (J. M. Lopez, A. Dromerick & E. Freese, unpublished results).

In the present paper, our aim was to investigate whether nitrofurantoin affects purine metabolism in the same manner as is observed in situations that initiate sporulation.

Table 1. *Bacterial strains used*

Strain	Properties	Origin
<i>Bacillus subtilis</i> 61831	<i>lys trpC2 rel</i> ⁺	Br16 of J. Gallant
<i>Bacillus subtilis</i> 61852	<i>lys trpC2 relA</i>	Br17 of J. Gallant
<i>Escherichia coli</i> CP78	<i>his leu arg thr thi relA</i> ⁺	D. Richter
<i>Escherichia coli</i> CP79	<i>his leu arg thr thi relA cca</i>	D. Richter
<i>Serratia marcescens</i> HY		Our collection

METHODS

Abbreviations. ppGpp, guanosine 3'-diphosphate 5'-diphosphate; pppGpp, guanosine 3'-diphosphate 5'-triphosphate.

Bacterial strains and growth conditions. The strains used and their properties are listed in Table 1. Liquid cultures were inoculated to give an absorbance (A_{578}) of less than 0.1 with cells grown overnight on Tryptose Blood Agar Base plates (Difco), and were shaken at 37 °C. The A_{578} was monitored in cuvettes with 1 cm light path. In all experiments, the growth medium had the following composition: 10 mM-(NH₄)₂SO₄, 2 mM-MgCl₂, 0.7 mM-CaCl₂, 50 µM-MnCl₂, 5 µM-FeCl₃, 1 µM-ZnCl₂, 20 mM-morpholinopropanesulphonate (pH adjusted to 7.0 with KOH), 1% (w/v) vitamin-free Casamino acids (Difco), and 20 mM-glutamic acid (pH adjusted to 7.0 with KOH). The amount of phosphate in the Casamino acids made the phosphate concentration in the medium 1 mM. Potassium phosphate was added to bring the final phosphate concentration to 2 mM.

Determination of nucleotides. [³²P]Phosphate [300 µCi ml⁻¹, 50 µCi (µmol P)⁻¹; carrier-free; Amersham/Buchler] was added to the cultures at an A_{578} of 0.15. After the A_{578} had reached 0.75, 50 µl amounts were withdrawn at intervals with an automatic pipette and rapidly added to ice-cold microfuge vials each containing 3 µl concentrated formic acid. After standing for 30 min in ice, the extracts were centrifuged and 5 µl samples of the supernatants were applied to a polyethyleneimine-cellulose thin-layer plate (0.1 mm thickness, Polygram CEL 300 PEI; Macherey-Nagel, Düren, F.R.G.). The plates were developed in the first dimension in 0.1 M-lithium citrate, pH 5.0, dried at room temperature, and washed for 15 min in absolute methanol. After drying, the plates were developed in the second dimension in 1.5 M-KH₂PO₄/K₂HPO₄, pH 3.5, dried, placed against an X-ray film (Curix RPI; Agfa-Gevaert) and incubated overnight. The radiation of the ³²P was intensified using intensifying screens (Cronex Xtra Life, DuPont Co.) at -70 °C. After development of the X-ray films (using Kodak X-ray developer), the radioactive spots in the thin-layer plates were located, cut out, and counted in a liquid scintillation counter in 10 ml toluene scintillator. Non-radioactive standards (5 µg) were always co-chromatographed in order to identify the radioactive spots. The results are expressed in pmol (A_{578} unit)⁻¹ (ml culture)⁻¹.

For the experiment described in Fig. 4, the radioactive extracts were applied 3 cm from the bottom of the thin-layer plate. To remove the inorganic phosphate, the plates were first developed in the reverse direction, with 0.1 M-lithium citrate, pH 5.0, running towards the bottom of the plate. This treatment moved the inorganic phosphate approximately 2 cm from the origin, without significantly changing the positions of ppGpp, pppGpp or GTP. After drying, a strip approximately 2 cm wide, containing the inorganic phosphate, was cut off the bottom of the plate. The remainder of the plate was washed in absolute methanol for 15 min, dried, and developed in the normal direction in 1.5 M-potassium phosphate, pH 3.5. The removal of the P_i minimized streaking and allowed a better quantification of ppGpp and pppGpp.

Synthesis of RNA and protein. Samples of 1.6 ml were withdrawn from the cultures at the appropriate times and mixed with 1.6 ml of ice-cold 10% (w/v) trichloroacetic acid. After standing for 30 min or longer in ice, 1 ml samples of the extracts were filtered through membrane filters (Schleicher & Schüll, BA85; 0.45 µm pore size) and the filters were each washed twice with 5 ml 5% (w/v) trichloroacetic acid (0 °C). The filters were dried at room temperature and counted in a liquid scintillation counter using 10 ml of Bray's scintillator. This procedure was repeated three times for each sample extract. The average of the three values was used to calculate the c.p.m. (ml culture)⁻¹.

Chemicals. Nitrofurantoin was obtained from Röhm-Pharma (Darmstadt, F.R.G.); ppGpp, pppGpp and the other nucleotides used as standards were purchased from ICN (Plainview, N.Y., U.S.A.).

RESULTS AND DISCUSSION

The stringent response of B. subtilis after addition of nitrofurantoin

After addition of nitrofurantoin (80 µM) to a culture of the stringent *B. subtilis* strain 61831, the intracellular concentrations of ppGpp, pppGpp and ATP increased, while the concentration of GTP decreased (Fig. 1). Within 30 min the concentrations of ppGpp, pppGpp and ATP had returned to the values observed before the drug addition, whereas that

Fig. 1. Changes in the intracellular nucleotide concentrations after treatment of the stringent and relaxed strains of *B. subtilis* with nitrofurantoin. To cultures growing exponentially in synthetic medium containing $2 \text{ mM-}[^{32}\text{P}]\text{P}_i$ ($300 \mu\text{Ci ml}^{-1}$), nitrofurantoin was added ($80 \mu\text{M}$) when the A_{578} reached 0.75. At intervals, samples were withdrawn from the cultures and extracted with formic acid; the nucleotides present in the extracts were quantified as described in Methods. Open symbols indicate results for the stringent strain 61831, and closed symbols those for the relaxed strain 61852: $\triangle$, $\blacktriangle$, ppGpp; ∇ , $\blacktriangledown$, pppGpp; $\circ$, $\bullet$, ATP; $\square$, $\blacksquare$, GTP.

of GTP recovered more slowly and had still not reached its original value after 60 min. Higher concentrations of nitrofurantoin, such as are needed for the initiation of sporulation, caused a longer lasting increase in the concentrations of ppGpp and pppGpp and a decrease in that of GTP with no recovery or a very sluggish recovery lasting several hours (not shown). As shown previously (Lopez *et al.*, 1979), the longer a GTP deficiency lasts the more cells will sporulate, the number of spores formed reaching a maximum after 4 h and then remaining constant if the deficiency is prolonged beyond this time.

With the isogenic relaxed strain 61852, no ppGpp or pppGpp accumulated; instead, the concentration of both ATP and GTP declined (Fig. 1). Sporulation of this strain after the addition of nitrofurantoin has not yet been studied.

RNA and protein accumulation in the stringent and relaxed strains of B. subtilis after exposure to nitrofurantoin

During the stringent response, however provoked, 'normal' stringent cells undergo a variety of changes in the rates of synthesis and degradation of cell polymers (for review, see Gallant, 1979). The rate of RNA accumulation is severely curtailed in the stringent strains (Sands & Roberts, 1952), whereas RNA continues to accumulate for a while in the strains with the relaxed phenotype. We wanted to know whether the inhibitory effect of nitrofurantoin on RNA accumulation in *B. subtilis* could be explained by the ability of the drug to elicit the stringent response, or whether additional modes of action needed to be postulated. For this purpose, we determined the rate of RNA accumulation in both the stringent and the relaxed strains, by measuring the incorporation of $[^3\text{H}]\text{uracil}$ into acid-precipitable material. Nitrofurantoin ($80 \mu\text{M}$) severely reduced the rate of RNA accumulation in the stringent strain 61831 (Fig. 2*a*), whereas the rate of RNA accumulation in the nitrofurantoin-treated culture of the relaxed strain 61852 remained similar to that of the control culture (Fig. 2*b*). This indicates that the inhibition of RNA synthesis caused by nitrofurantoin is mainly a consequence of the stringent response prompted by the drug.

Fig. 2. Effect of nitrofurantoin on the rate of RNA synthesis in stringent and relaxed strains of *B. subtilis*. Cultures were grown until the A_{578} reached 0.75, and then $[5\text{-}^3\text{H}]\text{uracil}$ [$0.5\text{ }\mu\text{Ci ml}^{-1}$; 25 mCi mmol^{-1}] was added. The cultures were immediately divided: one half received $80\text{ }\mu\text{M}$ -nitrofurantoin; the other served as a control. At intervals, samples were withdrawn from the cultures, and the incorporation of uracil into acid-precipitable material was determined as described in Methods. (a) Stringent strain 61831 and (b) relaxed strain 61852: $\circ$, nitrofurantoin-treated culture; $\bullet$, control culture.

Fig. 3. Effect of nitrofurantoin on the rate of protein synthesis in stringent and relaxed strains of *B. subtilis*. $\text{L-}[U\text{-}^{14}\text{C}]\text{Valine}$ ($0.5\text{ }\mu\text{Ci ml}^{-1}$) was added to exponentially growing cultures. The cultures were immediately divided: one half received $80\text{ }\mu\text{M}$ -nitrofurantoin; the other served as a control. At intervals, samples were withdrawn from the cultures, and the incorporation of valine into acid-precipitable material was determined as described in Methods. (a) Stringent strain 61831 and (b) relaxed strain 61852: $\circ$, nitrofurantoin-treated culture; $\bullet$, control culture.

An analogous experiment in which, instead of uracil, $[^{14}\text{C}]\text{valine}$ was added to the medium (which already contained the amount of valine present in the Casamino acids) showed that nitrofurantoin inhibited the rate of protein accumulation to the same degree in both the stringent and the relaxed strains (Fig. 3).

Fig. 4. Effect of nitrofurantoin on the formation of ppGpp and pppGpp in stringent and relaxed strains of *B. subtilis* and *E. coli*, and in a stringent *S. marcescens* strain. Cultures of the different strains were treated as described in the legend to Fig. 1. Samples were extracted prior to the addition of nitrofurantoin (designated -NF) and 15 min thereafter (+NF). The extracts were chromatographed on a polyethyleneimine-cellulose thin-layer plate. The figure shows an autoradiograph of the thin-layer plate.

Thus, the inhibition of protein synthesis was a direct effect of nitrofurantoin, but the inhibition of RNA synthesis, in contrast, was a consequence of the stringent response which had been simultaneously elicited. The drug either interferes with the biosynthesis of one or several amino acids or it inhibits the charging of one or several tRNA species thus creating a ribosomal A-site loaded with an uncharged tRNA, a condition which prompts the stringent response (Haseltine & Block, 1973).

For the purposes of this paper, it is important that in *B. subtilis* nitrofurantoin causes ppGpp to accumulate and the concentration of GTP to decrease to a concentration which, if reached by other means (for example by the addition of inhibitors of the GTP pathway) is sufficiently low to initiate sporulation. In addition, these experiments demonstrate that the inhibition of RNA accumulation caused by nitrofurantoin is mediated by the stringent response.

Increase in the intracellular concentration of ppGpp in E. coli and S. marcescens after nitrofurantoin addition

Nitrofurantoin, like many other nitrofurans, enjoys wide therapeutic application because of its broad spectrum of antimicrobial activity. We therefore examined two Gram-negative

organisms, *Escherichia coli* and *Serratia marcescens*, to see whether they also increase their intracellular ppGpp concentration on treatment with nitrofurantoin. The experimental conditions were the same as described in the first experiment with *B. subtilis*. As shown in Fig. 4, ppGpp, but not pppGpp, accumulated significantly in the stringent *E. coli* strain, and only slightly in the isogenic relaxed strain; the stringent *S. marcescens* strain also accumulated ppGpp on nitrofurantoin treatment, but the accumulation was less than in *E. coli*.

In *E. coli* an increase in ppGpp without a concomitant increase in pppGpp is normally observed after a carbon shift-down (Gallant & Lazzarini, 1976) and is apparently independent of a functional *relA* gene product, since *relA* strains also accumulate ppGpp under these conditions. After treatment with nitrofurantoin, the relaxed *E. coli* strain produced much less ppGpp than the stringent strain. Therefore, a functional *relA* gene product seems to be involved in the mechanism of the ppGpp increase imposed by nitrofurantoin. This question, and others concerning the mode of action of nitrofurantoin responsible for the initiation of the stringent response and the induction of sporulation in *B. subtilis* (Fortnagel, 1981), are currently under investigation.

We wish to thank Anne Böhn for excellent technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

This paper is dedicated to Professor G. Pfeleiderer of the University of Stuttgart on his 60th birthday.

REFERENCES

- ANON. (1976). New animal drug applications: furaladone (NF 260), nitrofurazone (NF 73), nihydrazone (NF 64) – opportunity for hearing on proposal to withdraw approval of certain new animal drug applications. *Federal Register* **41**, 38884–34921.
- FORTNAGEL, P. (1981). Nitrofurans can partially overcome the glucose repression of sporulation. In *Spores VIII*. Edited by R. S. Losick, D. Tipper & A. L. Sonenshein. Washington, D.C.: American Society for Microbiology. (in the Press).
- FREESE, E., HEINZE, J. E. & GALLIERS, E. M. (1979). Partial purine deprivation causes sporulation of *Bacillus subtilis* in the presence of excess ammonia, glucose and phosphate. *Journal of General Microbiology* **115**, 193–205.
- GALLANT, J. A. (1979). Stringent control in *E. coli*. *Annual Review of Genetics* **13**, 393–415.
- GALLANT, J. A. & LAZZARINI, R. (1976). The regulation of ribosomal RNA synthesis and degradation in bacteria. In *Protein Synthesis: A Series of Advances*, pp. 309–359. Edited by E. McConkey. New York: Dekker.
- GRUNBERG, E. & TITSWORTH, E. H. (1973). Chemotherapeutic properties of heterocyclic compounds: monocyclic compounds with five-membered rings. *Annual Review of Microbiology* **27**, 317–346.
- HAMILTON-MILLER, J. M. T. & BRUMFITT, W. (1976). The versatility of nitro compounds. *Antimicrobial Agents and Chemotherapy* **2**, 5–7.
- HASELTINE, W. A. & BLOCK, R. (1973). Synthesis of guanosine tetra- and penta-phosphate requires the presence of a codon-specific uncharged transfer ribonucleic acid in the acceptor site of ribosomes. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 1564–1568.
- HEINZE, J. E., MITANI, T., RICH, K. E. & FREESE, E. (1978). Induction of sporulation by inhibitory purines and related compounds. *Biochimica et biophysica acta* **521**, 16–26.
- HERRLICH, P. & SCHWEIGER, M. (1976). Nitrofurans, a group of synthetic antibiotics with a new mode of action: discrimination of specific messenger RNA classes. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 3386–3390.
- LOPEZ, J. M., MARKS, C. & FREESE, E. (1979). The decrease of guanine nucleotides initiates sporulation of *Bacillus subtilis*. *Biochimica et biophysica acta* **587**, 238–252.
- LOPEZ, J. M., OCHI, K. & FREESE, E. (1981). Initiation of *Bacillus subtilis* sporulation caused by the stringent response. In *Spores VIII*. Edited by R. S. Losick, D. Tipper and A. L. Sonenshein. Washington, D.C.: American Society for Microbiology. (in the Press).
- MCCALLA, D. R. (1979). Nitrofurans. In *Antibiotics V, Part 1: Mechanism of Action of Antibacterial Agents*, pp. 176–213. Edited by F. E. Haun. New York: Springer.
- MITANI, T., HEINZE, J. E. & FREESE, E. (1977). Induction of sporulation in *Bacillus subtilis* by decoyinine and hadacidine. *Biochemical and Biophysical Research Communications* **77**, 1118–1125.
- SANDS, M. K. & ROBERTS, R. B. (1952). The effects of a tryptophan-histidine deficiency in a mutant of *Escherichia coli*. *Journal of Bacteriology* **63**, 505–511.
- TU, Y. & MCCALLA, D. R. (1976). Effect of nitrofurazone on bacterial RNA and ribosome synthesis and on the function of ribosomes. *Chemico-Biological Interactions* **14**, 81–91.