Stringent Control of Ribonucleic Acid Synthesis in *Bacillus subtilis* Treated with Granaticin

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1. The antibiotic granaticin interferes in *Bacillus subtilis* with the charging process of tRNA^{Leu} causing both the arrest of protein synthesis and bacteriostasis [A. Ogilvie, K. Wiebauer & W. Kersten (1975) *Biochem. J.* 152, 511–515]. A concomitant inhibition of RNA synthesis is observed. This inhibition was studied with mutant strains of *B. subtilis*. 2. Granaticin inhibits protein and RNA synthesis in stringently controlled *B. subtilis* (*rel*⁺) to about the same extent. In a relaxed mutant strain (*rel*[−]) of *B. subtilis*, protein synthesis is also inhibited, but the accumulation of RNA continues after the addition of the drug. 3. Chloramphenicol, which is known to abolish the stringent control mechanism, added simultaneously with granaticin, allows the synthesis of RNA to proceed in the stringent strain. 4. Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) accumulate in granaticin-treated stringently controlled *B. subtilis* but not in the *rel*[−] mutant. 5. It is concluded that the inhibition of RNA synthesis by granaticin can adequately be explained as a stringent response caused by the interference by the drug with leucyl-tRNA synthetase.

The signal for the stringent control of RNA synthesis in bacteria deprived of an amino acid is an uncharged tRNA in the acceptor site of the ribosome (Pederson *et al.*, 1973; Haseltine & Block, 1973). In previous reports (Ogilvie *et al.*, 1975*b*) we showed that in *Bacillus subtilis* cells treated with granaticin the aminoacylation of tRNA^{Leu} was specifically and drastically decreased, thus creating the condition for the stringent control mechanism (Edlin & Broda, 1968; Ryan & Borek, 1971). It was concluded that the primary action of granaticin is inhibition of leucyl-tRNA synthetase (EC 6.1.1.4) causing the arrest of protein synthesis. We have suggested that the concomitant inhibition of RNA accumulation in granaticin-treated cells could be adequately explained by such a 'stringent response'. An additional direct effect of granaticin on RNA synthesis has not been ruled out. Therefore the mode of action of the antibiotic in a regulatory mutant (*rel*[−]) of *B. subtilis* was compared with that in the wild-type strain (*rel*⁺). We now present evidence to show that, at low concentrations of granaticin, the inhibition of RNA accumulation is merely a consequence of this regulatory mechanism governed by the *rel* gene. A preliminary report of some of these results has appeared (Ogilvie *et al.*, 1974).

Materials and Methods

Granaticin was a gift from Dr. H. Zähner, Tübingen University, Tübingen, W. Germany. L-Phenylalanine,

uridine, ATP, GTP and chloramphenicol were obtained from Boehringer, Mannheim, W. Germany. Salt (reagent grade) for buffers and polyethyleneimine (PEI)-cellulose-coated t.l.c. glass plates were from E. Merck, Darmstadt, W. Germany and filters were from Sartorius Membranfilter G.m.b.H., Göttingen, W. Germany. [³H]Uridine (6.4 Ci/mmol), [2-¹⁴C]-uracil (40.6 mCi/mmol), [U-¹⁴C]phenylalanine (522 mCi/mmol) and [³²P]PO₄^{3−} (original specific radioactivity 50–100 Ci/mg of P) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Bacterial strains and culture conditions

The strains used were *B. subtilis* W23 (wild-type), *B. subtilis* BR16 RC^{str} and BR16 RC^{rel}, isogenic except for the *rel* locus and originally prepared by Swanton & Edlin (1972). The strains were kindly supplied by Dr. M. Swanton (University of California, Davis, Calif., U.S.A.). The BR strains require lysine and tryptophan for growth. The cells were grown in phosphate-buffered glucose-salts medium (Ogilvie *et al.*, 1975*a,b*). For experiments with [³²P]phosphate a medium with a low (0.25 mM) concentration of phosphate was used; it contained per litre: 6.05 g of Tris, 2.0 g of (NH₄)₂SO₄, 2.3 g of sodium glutamate, 0.33 mg of MnSO₄, 0.2 g of MgSO₄·7H₂O, 4 g of glucose, 90 mg of lysine, 80 mg of tryptophan and 57 mg of K₂HPO₄ and was adjusted to pH 7.0. The cells were continuously aerated by vigorous shaking on a rotary shaker at 37°C. Growth of the cultures was followed

photometrically at 578nm with an Eppendorf photometer. All experiments were carried out during steady exponential growth.

Measurements of RNA and protein synthesis

The incorporation of [^3H]uridine and [^{14}C]phenylalanine into acid-insoluble material was used to follow RNA and protein synthesis as described in detail by Ogilvie *et al.* (1975a). The orcinol method (Schneider, 1945) was used to measure the accumulation of RNA. From dual-labelled cultures 5ml samples were withdrawn and pipetted into 0.5ml of ice-cold trichloroacetic acid (50%). The suspension was whirled and portions (0.1ml) were withdrawn, filtered on membrane filters, washed, dried and counted for radioactivity to determine the incorporation of [^3H]uridine and [^{14}C]phenylalanine into RNA and protein respectively. The rest of the suspensions were centrifuged and washed with 3ml of trichloroacetic acid (5%). The sediment was resuspended with 0.2ml of NaOH (0.5M), 1ml of water and 1.2ml of orcinol reagent. The mixture was kept in boiling water for 30min, cooled, and the E_{660} determined. A solution (0.4mg/ml) of yeast RNA obtained from Boehringer was used as a standard. An extinction of 0.5 equalled 0.190mg of RNA.

MAK chromatography

Chromatography of nucleic acids on methylated albumin-coated Kieselgur (MAK) was performed as originally described by Sibatani & Mizuno (1963). The nucleic acids, labelled with [^{14}C]uracil, were extracted with ice-cold phenol after the frozen cells had been ground with purified sand (Kersten *et al.*, 1967).

Determination of the nucleotide pool

The incorporation of [^{32}P]orthophosphate into ATP, GTP and the MS ('magic spot') nucleotides guanosine tetraphosphate and guanosine pentaphosphate was determined as described by Cashel (1969). A 50 μl sample of the culture was mixed with 100 μl of 1.5M-formic acid containing ATP and GTP (0.1mg/ml each) as markers. After 20min in ice the samples were centrifuged in the cold and 5 or 10 μl of the supernatant was spotted on polyethyleneimine-cellulose-coated glass plates. The plates were developed in 1.5M- or 1.25M- KH_2PO_4 (pH3.4). The R_F values of the various nucleotides were identical with those given by Cashel (1969). Radioautography, scraping off the plates and determination of the relative nucleotide concentrations were performed as described by Cashel (1969). $^{32}\text{PO}_4^{3-}$ was added to a growing cell culture at an E_{578} of about 0.250. The nucleotide pool was fully labelled within about 15min when it reached a constant ratio of ^{32}P radioactivity per unit absorbance.

Results

Inhibition of RNA accumulation in wild-type *B. subtilis*

In cultures of *B. subtilis*, granaticin (0.1–0.2 $\mu\text{g/ml}$) decreases the rate of incorporation of [^3H]uridine more markedly than it does the rate of incorporation of [^{14}C]phenylalanine (Fig. 1). About 10–30min after the addition of the drug, the incorporation of the precursors is equally inhibited. Since granaticin might somehow affect the uptake of uridine the question was examined of whether the incorporation of [^3H]uridine into acid-precipitable material really reflects the accumulation of stable RNA. The amount

Fig. 1. Inhibition of RNA synthesis in *B. subtilis* W23 by granaticin

An exponentially growing culture (70 ml) was labelled with [^3H]uridine (1 $\mu\text{Ci/ml}$; 62.5 $\mu\text{Ci/mmol}$). Granaticin (0.15 $\mu\text{g/ml}$) was added at an E_{578} of the culture of about 0.5, as indicated by the arrows. Samples (5 ml) were withdrawn and pipetted into ice-cold trichloroacetic acid (50%). The incorporation into acid-precipitable ^3H radioactivity was determined with 0.1 ml samples (a). The rest of the precipitate was used to determine the RNA content with orcinol reagent as described in the Materials and Methods section (b). ●, Growing culture; ○, granaticin-inhibited culture.

Table 1. Inhibition of the synthesis of tRNA, 16S and 23S rRNA in *B. subtilis* treated with granaticin

Growing cultures of *B. subtilis* W23 (200 ml; E_{578} about 0.7) were treated with granaticin at various concentrations. After 5 min [^{14}C]uracil (0.05 $\mu\text{Ci/ml}$; 50 $\mu\text{Ci/0.1 mmol}$) was added, and 20 min later the cultures were quickly cooled and centrifuged. RNA was extracted and chromatographed as described in the Materials and Methods section. The ratio of radioactivity/ E_{260} unit was determined for the pooled fractions of tRNA, 16S and 23S RNA.

Granaticin ($\mu\text{g/ml}$)	Radioactivity/ E_{260} unit			% inhibition
	tRNA	16S RNA	23S RNA	
0	1548	1607	1569	0
0.1	1423	1278	1280	10-20
0.2	948	891	875	35-45
0.3	102	92	87	90-95

of RNA was measured by the orcinol method simultaneously with the incorporation of [^3H]uridine. In cells treated with granaticin (see Fig. 1) the incorporation of [^3H]uridine reflects the accumulation of RNA as measured by the orcinol method.

The synthesis of tRNA and rRNA was inhibited by granaticin to almost the same extent (Table 1). The experiments were performed at higher cell density. To get an inhibition of RNA synthesis comparable with that in the other experiments adequate concentrations of granaticin were used.

Effect on RNA and protein synthesis in *B. subtilis* rel^+ and rel^-

The stringent control mechanism is governed by the *rel* gene (Stent & Brenner, 1961; Ryan & Borek, 1971) and mutation in this locus provides 'relaxedness'. In the relaxed (rel^-) mutant strains RNA accumulation can proceed in spite of an amino acid restriction. We tested a pair of *B. subtilis* strains (BR16 $\text{RC}^{\text{str}}=rel^+$; BR16 $\text{RC}^{\text{rel}}=rel^-$), isogenic except for the *rel* gene. On treatment with granaticin (0.2 $\mu\text{g/ml}$), RNA accumulation proceeds in the relaxed mutant (Fig. 2) but immediately stops in the stringent cells (Fig. 3). The synthesis of protein is equally inhibited in both strains by granaticin, but the relaxed strain reacts after a slight delay. A tardy response seems to be typical for relaxed strains when the required amino acid is restricted (Sokawa *et al.*, 1971). From these results it is evident that granaticin inhibits RNA synthesis only in those cells with intact control of RNA synthesis.

Effect of chloramphenicol on granaticin-treated stringent cells

When protein synthesis is inhibited by antibiotics acting at the ribosomal level, e.g. by chloramphenicol,

Fig. 2. Effect of granaticin on RNA (a) and protein (b) synthesis in *B. subtilis* BR16 RC^{str}

An exponentially growing culture was labelled with [^3H]uridine (5 $\mu\text{Ci/ml}$; 62.5 $\mu\text{Ci/mmol}$) and [^{14}C]phenylalanine (0.25 $\mu\text{Ci/ml}$; 2 mCi/mmol). Granaticin (0.2 $\mu\text{g/ml}$) was added at an E_{578} of the culture of about 0.3, as indicated by the arrows. Samples (0.1 ml) were withdrawn and the acid-precipitable radioactivity was determined. ●, Growing culture; ○, culture after the onset of granaticin inhibition.

the synthesis of RNA is not affected (Edlin & Broda, 1968; Cashel, 1969). Moreover, stringent cells starved of an amino acid continue to accumulate RNA after additional treatment with chloramphenicol. Thus chloramphenicol abolishes the stringent control of RNA synthesis (Edlin & Broda, 1968; Cashel, 1969; Lund & Kjelgaard, 1972). When chloramphenicol is added to stringent *B. subtilis* cells simultaneously with granaticin, the incorporation of [^3H]uridine

Fig. 3. Effect of granaticin on RNA (a) and protein (b) synthesis in *B. subtilis* BR16 RC^{rel}

The dual-labelled culture was treated in a manner similar to that described in the legend of Fig. 2. ●, Growing culture; ○, culture after the onset of granaticin inhibition.

is not inhibited whereas the incorporation of $[^{14}\text{C}]\text{phenylalanine}$ stops completely (Fig. 4). The antibiotic also nullifies the stringent control triggered by granaticin.

MS nucleotides

A well-established feature of the stringent response to restriction of an amino acid is the accumulation of two unusual guanosine nucleotides: guanosine 5'-diphosphate-3'-diphosphate (ppGpp) and a pentaphosphate of guanosine (pppGpp) (Cashel & Gallant, 1969; Cashel, 1969; Cashel & Kalbacher, 1970; Haseltine *et al.*, 1972). Relaxed mutants fail to produce higher amounts of MS nucleotides on amino

acid deprivation, but are able to accumulate them during carbon shift-down conditions (Lazzarini *et al.*, 1971). In stringent cells, a sharp decrease in the GTP pool is observed concomitant with the occurrence of ppGpp (Edlin & Broda, 1968; Cashel, 1969) whereas the ATP pool decreases much less, if at all (Edlin & Broda, 1968). The kinetics of $[^{32}\text{P}]\text{phosphate}$ labelling of purine nucleotides were compared in the two *B. subtilis* strains (BR16 RC^{str} ,

Fig. 4. Effect of chloramphenicol on RNA (a) and protein (b) synthesis in granaticin-inhibited *B. subtilis* BR16 RC^{str}

At $E_{578} = 0.3$, a dual-labelled culture of *B. subtilis* RC^{str} was split into 2×1 ml portions. To one part was added granaticin ($0.2 \mu\text{g/ml}$); to the other granaticin and chloramphenicol ($100 \mu\text{g/ml}$) were added simultaneously at the time indicated by the arrows. Samples were withdrawn and acid-insoluble radioactivity was determined as outlined in the legend of Fig. 2. ●, Growing culture; ○, culture after the onset of granaticin inhibition; □, culture after the simultaneous addition of granaticin and chloramphenicol.

Fig. 5. MS nucleotide accumulation in granaticin-inhibited *B. subtilis* BR16 RC^{str}

B. subtilis RC^{str} was grown in Tris-buffered glucose-salt medium containing phosphate at a concentration of 0.25 mM. [³²P]Orthophosphate was added (200 μ Ci/ml), at an E_{578} of about 0.250. The arrows indicate the time at which granaticin was added (0.2 μ g/ml). Samples (50 μ l) were withdrawn to determine the relative contents of ATP, GTP and MS nucleotides as described in the Materials and Methods section. (a) shows ³²P radioactivity appearing in ppGpp, (b) shows ³²P radioactivity appearing in pppGpp and (c) shows the labelling of ATP and GTP. ●, Growing culture; ○, culture after the onset of granaticin inhibition.

BR16 RC^{str}) after the addition of granaticin. The results obtained with the stringent strain are shown in Fig. 5. The increase in ppGpp begins very shortly after the addition of granaticin and reaches a maximum within a few minutes. The amount of ATP also increases after the addition of granaticin. A primary effect of granaticin on oxidative phosphorylation can thus be excluded. The sharp decrease in the amount of GTP is almost certainly a consequence of the formation of MS nucleotides. Guanosine pentaphosphate accumulates to the same concentration as that of ppGpp. This ratio of ppGpp/pppGpp (about 1:1) is typical for *B. subtilis* (Gallant & Margason, 1972; Swanton & Edlin, 1972) and contrasts with the ratio of 3:1 observed in *E. coli* during amino acid starvation (Cashel & Gallant, 1969). In the relaxed mutant of *B. subtilis*, MS nucleotides did not increase over the control value after the addition of even higher concentrations of granaticin (0.4 μ g/ml). Thus the patterns of ATP, GTP and MS nucleotides, after the application of granaticin, are similar to those observed during starvation of an amino acid.

Conclusion

Granaticin affects RNA synthesis only in stringently controlled cells, and this effect can be abolished by chloramphenicol. The increase in guanosine tetraphosphate and guanosine pentaphosphate and the decrease in the GTP pool in granaticin-treated cells are identical with the effects observed during amino acid starvation. From these results we conclude that at low concentrations granaticin has no direct effect on the synthesis of RNA in *B. subtilis*, but that the inhibition of RNA synthesis is merely a consequence of the stringent control mechanism which is due to the primary action of granaticin on leucyl-tRNA synthetase.

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References

- Cashel, M. (1969) *J. Biol. Chem.* **244**, 3133–3141
- Cashel, M. & Gallant, J. (1969) *Nature (London)* **221**, 838–841
- Cashel, M. & Kalbacher, B. (1970) *J. Biol. Chem.* **245**, 2309–2318
- Edlin, G. & Broda, P. (1968) *Bacteriol. Rev.* **32**, 206–226
- Gallant, J. & Margason, G. (1972) *J. Biol. Chem.* **243**, 2289–2294
- Haseltine, W. A. & Block, R. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1564–1568
- Haseltine, W. A., Block, R., Gilbert, W. & Weber, K. (1972) *Nature (London)* **238**, 381–384

- Kersten, W., Kersten, H., Steiner, F. E. & Emmerich, B. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 1415-1423
- Lazzarini, R. A., Cashel, M. & Gallant, J. (1971) *J. Biol. Chem.* **246**, 4381-4385
- Lund, E. & Kjeldgaard, N. O. (1972) *Eur. J. Biochem.* **28**, 316-326
- Ogilvie, A., Wiebauer, K. & Kersten, W. (1974) *Abstr. FEBS Meet. 9th*, Abstr. s4b9
- Ogilvie, A., Lämmermann, M., Wiebauer, K. & Kersten, W. (1975a) *Biochim. Biophys. Acta* **395**, 136-145
- Ogilvie, A., Wiebauer, K. & Kersten, W. (1975b) *Biochem. J.* **152**, 511-515
- Pedersen, F. S., Lund, E. & Kjeldgaard, N. O. (1973) *Nature (London) New Biol.* **243**, 13-15
- Ryan, A. M. & Borek, E. (1971) *Prog. Nucleic Acid Res. Mol. Biol.* **11**, 193-228
- Schneider, W. C. (1945) *J. Biol. Chem.* **161**, 293-303
- Sibatani, A. & Mizuno, N. (1963) *Biochim. Biophys. Acta* **76**, 188-200
- Sokawa, Y., Sokawa, J. & Kaziyo, Y. (1971) *Nature (London) New Biol.* **234**, 7-10
- Stent, G. S. & Brenner, S. (1961) *Proc. Natl. Acad. Sci. U.S.A.* **47**, 2005-2014
- Swanton, M. & Edlin, G. (1972) *Biochem. Biophys. Res. Commun.* **46**, 583-588