A study of bacterial response to polypeptide antibiotics

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When *Escherichia coli* cells are treated with either polymixin or gramicidin at concentrations that block protein and RNA synthesis, they accumulate a significant amount of guanosine tetraphosphate ppGpp. Such accumulation occurs in stringent (*relA*$^+$) as well as in relaxed (*relA*) strains and no guanosine pentaphosphate pppGpp is then detected within the cells. These observations suggest that polypeptide antibiotics elicit ppGpp formation through a mechanism different from the stringent control system triggered by amino acid starvation of bacteria. Experiments based on tetracycline action indicate, moreover, that the accumulation of ppGpp under polymixin or gramicidin treatment is connected with a strong restriction of the degradation rate of this nucleotide.

Polypeptide antibiotic *Polymixin* *Gramicidin* *Guanosine tetraphosphate*

1. INTRODUCTION

Polymixin B and gramicidin S are cyclic polypeptide antibiotics that bind to the cytoplasmic membrane of bacteria and modify its structural organization. Consequently, the cell envelope loses its properties as permeability barrier and a rapid release of cytoplasmic solutes occurs (review [1]). Although there is little doubt about the site of action of these drugs, the actual molecular mechanism responsible for their bactericidal action remains to be established [1]. In previous reports, evidence has been presented that the inhibition of bacterial growth is a result of the blockade of protein synthesis by the antibiotics [2,3]. Also, it has been shown that both DNA and RNA syntheses are strongly inhibited under drug treatment, and that active transport, respiration and cell wall synthesis are significantly reduced [4].

In this work, an attempt was made to investigate further the effects of polymixin and gramicidin on bacterial metabolism by measuring comparatively the levels of nucleotides in drug-treated and non-treated cells. Special attention was paid to guanosine tetraphosphate ppGpp, since this unusual nucleotide is likely to play a key role in the coordination of macromolecular synthesis [5]. Namely, it accumulates in stringent bacteria of *relA*$^+$ genotype during amino acid starvation when RNA and protein syntheses are concomitantly arrested. By contrast, it is absent in starving relaxed (*relA*) mutants which keep synthesizing RNA at near-normal rate while protein formation is blocked [6]. For this reason, a parallel analysis of the two types of strain was carried out in the present study.

The main result shows that polypeptide antibiotics elicit ppGpp accumulation in both stringent and relaxed strains of *Escherichia coli*. The increased level of this nucleotide within the cells is due to a strong restriction of its degradation rate.

2. MATERIALS AND METHODS

The otherwise isogenic stringent/relaxed pair of *E. coli* strains CP78 (*relA*$^+$) and CP79 (*relA*) was used [7]. Both strains require histidine, threonine, arginine and leucine for growth. Cells were cultured under forced aeration at 37°C in a

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minimal Tris–glucose medium [8] supplemented with the 4 essential amino acids (50 μg/ml each) and containing 1 mM KH_2PO_4. Starvation experiments were carried out by transferring exponentially growing bacteria to a fresh medium lacking arginine [8].

The synthesis of RNA and protein was measured by the incorporation of, respectively, [^{3}H]uracil and [^{14}C]proline into cold 5% trichloroacetic acid precipitates. Radioactivity was counted in a scintillation fluid using the appropriate double-label setting in a model 3380 Tri-Carb Packard spectrometer.

Nucleotide concentrations were determined by using the method in [9]. Briefly, [^{32}P]orthophosphate (170–200 μCi/ml) was added to exponentially growing cultures approximately one doubling before the earliest sample was taken. In starvation experiments, bacteria were collected on Millipore filters, then resuspended in an arginine-deprived fresh medium containing labeled orthophosphate at the same specific activity as before. For antibiotic treatment, drugs were added directly into the pre-labeled culture medium. Aliquots (1 ml each) were withdrawn at intervals of time and treated with an equal volume of 2 M formic acid for 30 min at 0°C. The suspension was subjected to low-speed centrifugation, then 10–20 μl formic acid extract were analyzed by ascending chromatography on poly(ethyleneimine) cellulose plates. After migration, the nucleotides (ATP, GTP, ppGpp and pppGpp) were localized by autoradiography using Kodak X-O-Mat films, and their respective concentrations were calculated and expressed as pmol/ml culture medium.

Polymixin B sulfate (7700 USP units/mg) and gramicidin S hydrochloride were purchased from Sigma Chemical Co. (St Louis MO). Tetracycline hydrochloride was obtained from Boehringer (Mannheim) and radioactive compounds from the French CEA. Other reagents were of analytical grade from Sigma Chemicals or Merck.

3. RESULTS

Control experiments were first performed to measure the effects of polymixin and gramicidin on protein and RNA synthesis. Strains CP78 and CP79 were treated separately with various doses of either antibiotic, and the amount of radioactive proline and uracil incorporated into the trichloroacetic acid-precipitable material was determined in each case. It was observed that 10 μg polymixin/ml or 200 μg gramicidin/ml promoted the same drastic inhibition, by >95%, of both protein and RNA syntheses in either type of strain (not shown). This finding was in agreement with other data already reported on the effects of polypeptide antibiotics on macromolecular synthesis [2–4]. Similar results had been obtained under amino acid starvation of the stringent strain CP78 [8]. However, a significant difference could be noted in the case of the relaxed mutant CP79. Indeed, in the absence of an amino acid essential to growth, RNA synthesis had been shown to continue at a near-normal rate while protein synthesis was blocked [8] whereas, as indicated above, RNA synthesis was found to be almost completely arrested during treatment with polymixin or gramicidin. This suggested that the effects of the two drugs on bacterial metabolism were basically different from those of amino acid deprivation. Therefore, the two types of effect were further analyzed in parallel.

Table 1

The effects of amino acid starvation and polypeptide antibiotic treatment on nucleotide levels in CP78 cells of *E. coli*

Treatment	Time (min)	Nucleotide (pmol/ml)			
		ATP	GTP	ppGpp	pppGpp
None		268	275	0	0
Amino acid starvation	10	138	185	154	34
	20	118	180	180	44
Polymixin	10	38	82	83	0
	20	36	40	88	0
Gramicidin	10	67	76	32	0
	20	66	51	32	0

Bacteria were grown in exponential phase in the presence of radioactive orthophosphate then either starved of arginine or treated with polymixin (10 μg/ml) or gramicidin (200 μg/ml). After 10 or 20 min, a formic acid extract was prepared in each case, and nucleotides were separated by ascending chromatography. They were detected by autoradiography, and their respective concentrations were determined and expressed as pmol/ml culture medium. Average values from 3–5 expt are given

The intracellular levels of nucleotides were measured in drug-treated or amino acid-starved bacteria and compared to those in exponentially growing cells. The results displayed in table 1 show that, as expected [5,6], ppGpp and pppGpp accumulate in the stringent strain during amino acid starvation while the ATP and GTP pools are decreased. Similarly, ppGpp accumulates under polymixin or gramicidin treatment, although to a lesser extent than in starving cells, and the size of nucleoside-triphosphate pools is also greatly reduced, especially in the presence of polymixin. However, no pppGpp is detected within the cells whatever the antibiotic used. The results reported in table 2 confirm that, in the relaxed strain, guanosine polyphosphates do not accumulate upon amino acid starvation [5,6], and the ATP/GTP concentration is only slightly modified. But it can be seen that, surprisingly enough, polypeptide antibiotics induce the accumulation of ppGpp, especially polymixin. Here again, no pppGpp is then present within the cells.

Such accumulation of ppGpp occurring under drug treatment was analyzed in more detail. The synthesis of the nucleotide in strain CP78 was first induced by arginine starvation then blocked by the addition of tetracycline [10,11] and, thereafter, its degradation was followed in the presence or absence of polypeptide antibiotic. The data presented in table 3 indicate that the presence of polymixin as well as gramicidin results in a pronounced increase of the biological lifetime of ppGpp after tetracycline addition. When either one of the two drugs was added alone to starving cells, an overaccumulation of ppGpp was obtained.

Table 2

The effects of amino acid starvation and polypeptide antibiotic treatment on nucleotide levels in CP79 cells of *E. coli*

Treatment	Time (min)	Nucleotide (pmol/ml)			
		ATP	GTP	ppGpp	pppGpp
None		213	220	0	0
Amino acid	10	176	220	0	0
starvation	20	160	205	0	0
Polymixin	10	47	115	36	0
	20	38	74	43	0
Gramicidin	10	102	161	16	0
	20	88	109	16	0

Experimental conditions were the same as those described in table 1

Table 3

Degradation of ppGpp in the presence of polypeptide antibiotics

Antibiotic treatment	ppGpp (pmol/ml)		
	3 min	5 min	9 min
Tetracycline	22	10	8
+ polymixin	140	107	98
+ gramicidin	116	93	60
Polymixin	255	314	197
Gramicidin	193	211	253

After arginine starvation for 20 min, cells of strain CP78 were treated (zero time) with 200 μg tetracycline/ml either alone or together with polymixin (10 μg/ml) or gramicidin (200 μg/ml). The intracellular concentration of ppGpp was then measured, in each case, as a function of time. The effects of the same doses of polymixin or gramicidin in the absence of tetracycline were also analyzed. The initial level of ppGpp, at zero time, was 180 pmol/ml

4. DISCUSSION

The main observation of this study is that polymixin and gramicidin induce the accumulation of ppGpp in bacteria when added at concentrations that block protein and RNA synthesis.

It seems likely that such cellular response to polypeptide antibiotics differs from that triggered by amino acid starvation. Indeed, the relaxed mutants that have lost the ability to produce ppGpp under amino acid deprivation still retain the ability to accumulate the nucleotide in response to drug treatment. In addition, RNA synthesis is almost totally inhibited in these mutants when treated with either polymixin or gramicidin, whereas it is not substantially affected by amino acid starvation. Furthermore, in both stringent and relaxed strains, guanosine pentaphosphate pppGpp, that is the physiological precursor of ppGpp in starving cells [12,13], is never detected in the presence of polypeptide antibiotics. It

therefore appears that the metabolism of ppGpp is then adjusted through a mechanism independent of the *relA* gene product, that is quite distinct from the stringent control system. This mechanism remains to be elucidated but it must involve, in particular, a strong reduction of the degradation rate of ppGpp, as revealed by the experiments based on the tetracycline action and those showing that the nucleotide concentration in starving cells is further elevated by either polypeptide antibiotic.

It is noteworthy that the bacterial cell responds in a similar way to different physiological disturbances by accumulating ppGpp in all cases. This applies not only to several types of amino acid starvation [5,6] but also to temperature shock [14,15], treatment with dinitrophenol [16] or levallorphan [11], growth in hypertonic salt solutions [17] and, as shown here, treatment with polypeptide antibiotics. Therefore, ppGpp may be considered, in a rather general way, as a biological indicator of various environmental perturbations.

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