EFFECT OF FUSIDIC ACID ON SPORULATION OF BACILLUS SUBTILIS

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Received 26 October 1973

1. Introduction

The antibiotic fusidic acid has been found to block protein synthesis both in vivo and in vitro in bacterial [1–4] and eukaryotic cells [5, 6]. It was suggested that in bacterial cells the inhibition by this antibiotic is due to the formation of a GDP-ribosome-fusidic acid-EFG complex [7] which blocks the aminoacyl-tRNA binding to the A-acceptor site, thus preventing peptide chain elongation.

It was recently reported [8] that fusidic acid, when added to a log phase culture of *B. subtilis* (Marburg strain 60015), inhibits growth and spore development, whereas the addition of this antibiotic to stationary phase cells has no effect on sporulation capacity. This change in sensitivity to fusidic acid was interpreted by the authors to be the result of an alteration of ribosomal proteins in sporulating cells.

Investigating the possibility of an alteration in the protein synthesizing machinery of a thermosensitive sporulation mutant of *B. subtilis* [9], we also tried, among others, the effect of fusidic acid on this mutant grown at the restrictive (30°C) and the permissive (42°C) temperatures.

The results reported here clearly show that: i) fusidic acid inhibits protein synthesis in vivo, whatever the time of addition of this antibiotic might be, during growth or sporulation; ii) protein synthesis in vitro is also inhibited irrespective of the source of ribosomes: log phase or stationary phase cells, from sporulating or non-sporulating cultures; iii) fusidic acid affects spore development even when added 7 hr after termination of exponential growth (T_7). These results are in contradiction to those obtained by Fortnagel and Bergmann [8].

2. Materials and methods

2.2. Strain used

168 Wild-type; 168 trp^- thy^- and the thermosensitive-sporulation mutant ts-4 derived from it [10].

2.2. Media and growth conditions

Cells were grown in nutrient broth (Difco) [10]. When necessary tryptophan and thymine (50 μg/ml) were added to the medium.

Growth and sporulating were carried out in Fernbach flasks containing a volume of medium not exceeding one tenth the volume of the flask and incubated at the desired temperature. Growth was followed with a Zeiss spectrophotometer by measuring the absorbance at 650 nm. Spores were counted by plating the cells from a 24 hr culture after heating 10 min at 80°C. The end of exponential growth is designated as T_0 and T_1, T_2, etc. the time (in hours) after exponential growth has stopped.

2.3. Preparation of high salt washed ribosomes

About 2 g of fresh cells were suspended in 3 vol of buffer A (10 mM Tris, pH 7.4; 10 mM magnesium acetate; 60 mM ammonium chloride and 6 mM 2-mercapthothanol) and were lysed by addition of 0.5 mg/ml lysozyme. In some experiments ribosomes were prepared by grinding cells with alumina and extracted with buffer A. Ribosomal pellet obtained from 150 000 *g* centrifugation for 3 hr was suspended in buffer A and recentrifuged for 15 hr at 100 000 *g* through a cushion of 34% sucrose dissolved in buffer A. The

resulting pellet was washed with 3 cycles of suspension and centrifugation at 150000 *g* in buffer A containing 1 M ammonium chloride (high salt-buffer). The final pellet was suspended in buffer A at a concentration of 125–250 A_{260} U/ml and kept at −70°C in small fractions.

2.4. High speed supernatant (S150) preparation

The high speed supernatants were prepared by two successive centrifugations of cell lysates at 150000 *g* for 3 hr, dialyzed overnight against buffer B (10 mM Tris–HCl, pH 7.4; 10 mM magnesium acetate; 30 mM ammonium chloride; 0.1 mM dithiothreitol and 10% glycerol) and were stocked at −70°C at a concentration of about 15 mg/ml protein.

Only the preparations showing negligible contamination by ribosomes, as detected by ribosome-independent polyphenylalanine synthesis, were used for this study.

2.5. Poly U dependent polyphenylalanine synthesis

The assays were carried out according to the method of Hirashima et al. [11] with following modifications. Each tube contained approximately 1.3 A_{260}U ribosomes, 525 pmoles of [^{14}C]phenylalanine (2×10^5 cpm) and 150 μg of S150 protein in a total volume of 125 μl.

After incubation for 30 min at 30°C the reaction was stopped by addition of 1 ml cold 10% trichloroacetic acid, left 30 min in ice and then heated at 90°C for 20 min. The precipitate was collected and washed with 2.5% cold trichloroacetic acid followed by ethanol on glass fiber filters. The dried filters were counted in a toluene-based liquid scintillation medium.

Ribosome-dependent GTPase activity was determined by using ^{32}P γGTP according to the method of Nishizuka et al. [12]. Partially purified elongation factor G (EFG) from wild type log phase cells, prepared according to Leder [13] up to DEAE-Sephadex step, was used for this study. This EFG preparation contained about 7% elongation factor T (as assayed by elongation factor-dependent polyphenylalanine synthesis) and a negligible amount of ribosome-independent GTP splitting capacity. Washed ribosomes (1.6–2.2 A_{260}U per tube) did not have any significant contaminating GTPase activity.

3. Results

The addition of fusidic acid* (0.05 mM) to a log phase culture of *B. subtilis* 168 wild type stops [^{3}H]-methionine incorporation almost immediately. When the antibiotic was added at 3.5 hr after the end of exponential growth ($T_{3.5}$) a 70% inhibition of methionine incorporation was observed (fig. 1). The addition of fusidic acid to either log phase cells or to sporulating cells at T_7 inhibits spore formation by 100% and 80% respectively. A similar inhibitory response to fusidic acid was observed when the mutant strains (168 trp$^-$ thy$^-$ and ts-4) were used. Inhibition of spore formation by fusidic acid in these strains at $T_{3.5}$ is shown in table 1.

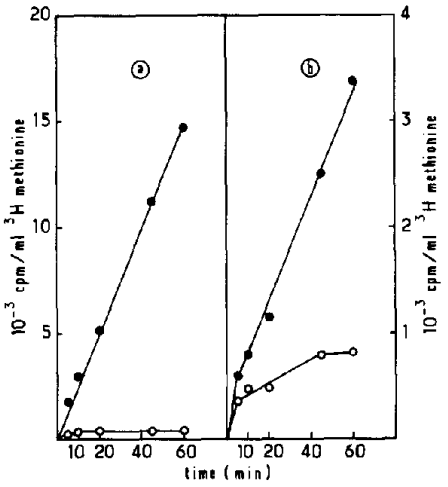

Fig. 1. Effect of 0.05 mM fusidic acid on the incorporation of [^{3}H]methionine during growth and sporulation of *B. subtilis* 168 wt. a) log phase; b) stationary phase ($T_{3.5}-T_{4.5}$). (●—●—●) control; (○—○—○) with fusidic acid; [^{3}H]methionine (0.5 μCi/ml) was added to a 10 ml culture within 30 sec after the addition of fusidic acid. Samples of 0.5 ml taken at the times indicated, were added to test tubes containing 2 ml cold 10% TCA and 1 mM cold methionine. The precipitates were heated at 90°C for 20 min, cooled, filtered on glass fiber filters, and washed with 2.5% TCA followed by 2 ml of ethanol. The radioactivity was counted in a scintillation counter using toluene based medium.

* Fusidic acid was a gift from Dr. W.O. Godtfredsen (Leo Pharmaceutical Laboratories, Denmark).

Table 1
Inhibition of spore formation in different strains by fusidic acid (0.05 mM).

Strains	Spores ml/culture		% Inhibition
	−Fusidic acid	+ Fusidic acid	
168 Wild type	1.25×10^8	5×10^6	96
ts-4	9.7×10^7	2.3×10^7	76
168 trp⁻thy⁻	2.5×10^8	4.3×10^7	83

The antibiotic was added at $T_{3.5}$.

3.1. Effect of fusidic acid on poly U-directed polyphenylalanine synthesis

Fig. 2 shows the inhibition, by fusidic acid, of the poly U-directed polyphenylalanine synthesis in presence of stationary phase ribosomes as a function of the concentration of the antibiotic. It can be seen that with ribosomal preparations from three different *B. subtilis* sporulating strains a 50% inhibition was obtained by about 0.2 mM fusidic acid and that it requires 3 mM to reach about 90% inhibition.

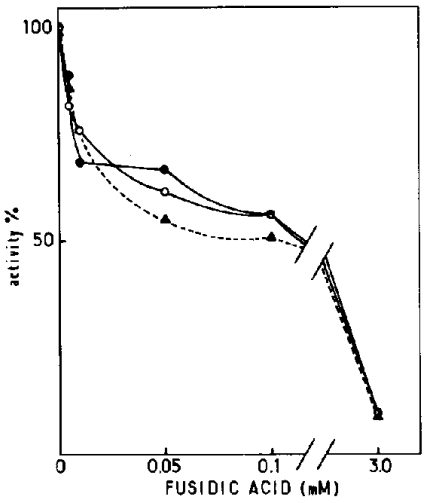

Fig. 2. Fusidic acid inhibition pattern of poly U-directed polyphenylalanine synthesis. Ribosomes were prepared from stationary phase (T_4) cells and supernatant (S150) was from log phase cells of the wild type. (●—●—●) ts-4 grown at 30°C; (o—o—o) ts-4 grown at 42°C; (▲--▲--▲) 168 trp⁻thy⁻ at 30°C. 100% activity represents 39 795, 33 792 and 13 260 cpm of [^{14}C]phenylalanine incorporated into protein with ribosomes from ts-4 at 30°C, 42°C and 168 trp⁻thy⁻ at 30°C, respectively.

Similar inhibition curves were obtained with ribosomes from log phase cells.

It was recognized that these experiments were carried out with high speed supernatant (S150) from log phase cells. In order to determine whether the stationary phase elongation factor G (EFG) has a different sensitivity towards fusidic acid, we also have assayed polyphenylalanine synthesis in the presence of unfractionated S150 (as a source of EFG) from stationary phase cells with homologous and heterologous ribosomes. The results are presented in table 2. It appears that in most cases a concentration of 0.05 mM fusidic acid inhibits phenylalanine incorporation regardless of whether stationary or log phase cells are used a a source of S150 supernatant, though this inhibition slightly decreased when stationary phase S150 was assayed in presence of homologous ribosomes. However, this difference disappeared at a concentration of 0.1 mM fusidic acid.

3.2. Ribosome-dependent GTPase activity

The effect of fusidic acid on EFG-dependent GTPase activity is shown in table 3. For the determination of this activity stationary phase ribosomes for either the 168 trp⁻thy⁻ strain or from the ts-4 mutant were used. It can be seen that an inhibition of about 50% was obtained by 0.1 mM fusidic acid. At lower concentrations of fusidic acid, inhibition varied with ribosomes from different sources, but it is always detectable at a concentration as low as 0.01 mM. These results clearly indicate that the ribosomes of the stationary phase cells remain sensitive to fusidic acid.

4. Discussion

The results reported here, using three strains of *B. subtilis*, clearly show that the antibiotic fusidic acid inhibits, during growth and stationary phase, the incorporation of labelled amino acids into acid precipitable material. Moreover, the sensitivity to fusidic acid is also retained in vitro, inhibiting the poly U-directed polyphenylalanine synthesis, irrespective of the source of washed ribosomes and high speed supernatant: sporulating cells or stationary phase cells from the thermo-sensitive ts-4 mutant grown at the restrictive temperature, at which sporulation is blocked.

Table 2
Inhibition of polyphenylalanine synthesis by fusidic acid (0.05 mM).

Source of S150	Source of ribosomes	[^{14}C]Phenylalanine incorporated (cpm)		% Inhibition
		−Fusidic acid	+Fusidic acid (0.05 mM)	
168 wt log phase	168 wt log phase	18 730	9 590	49
ts-4 at 30°C T_4	168 wt log phase	5 690	2460	57
ts-4 at 42°C T_4	168 wt log phase	6 200	2 995	51
ts-4 at 30°C T_4	ts-4 at 30°C T_4	16 140	10 700	34
ts-4 at 42°C T_4	ts-4 at 42°C T_4	14 620	9 150	37

Each test tube contained 150 μg of protein in the S150 prepared from the log phase or stationary phase cells.

Table 3
Inhibition by fusidic acid of the ribosome-dependent GTPase activity.

Fusidic acid (mM)	pmoles of ^{32}P liberated			
	168 trp$^-$thy$^-$ Grown at:		ts-4 Grown at:	
	30°C	42°C	30°C	42°C
0	168	322	252	378
0.01	150 (11)	265 (18)	170 (31)	182 (52)
0.05	–	250 (23)	133 (47)	210 (45)
0.1	75 (55)	155 (53)	130 (48)	100 (74)

To each test tube 16.75 μg of partially purified EFG was added. The activity is expressed as pmoles of ^{32}P liberated at 30°C in 10 min. The figures within parentheses are per cent inhibition. The activity in absence of elongation factor (0–5 pmole) was substracted from each figure.

Studies on fusidic acid resistant mutants of *E. coli* and *B. subtilis* indicate that the resistance trait is associated with elongation factor G and not with the ribosomes [14–16]. Furthermore, less binding of the antibiotic was observed when fusidic acid-resistant EFG was used for the fusidic acid–EFG–GDP–ribosome complex formation [14]. These observations led us to analyse the effect of fusidic acid on stationary phase cell high-speed supernatant (as a source of EFG). Here again our results show that the high-speed ribosome free supernatant from sporulating cells, like vegative cell supernatant, remains sensitive to fusidic acid.

The data presented here led us to the conclusion that fusidic acid inhibits protein synthesis, both in vivo and in vitro, in exponentially growing cells, or stationary phase cells irrespective of the spore phenotype of the strain. Our results are therefore different from those reported by Fortnagel and Bergmann [8] who observed that sporulating cells of *B. subtilis* M60015 lose sensitivity to fusidic acid. This resistance was interpreted by the authors as a reflection of a change in ribosomal proteins during sporulation. However, the lack of sensitivity of sporulating cells to fusidic acid in vitro was only based on experiments where unwashed stationary phase cell ribosomes were used as a source of GTPase activity in the presence of fusidic acid. Since unwashed ribosomes contain bound EFG, it is quite possible that the fusidic acid resistance of the particular strain used by the authors is due to an altered EFG.

Acknowledgements

This work was supported by grants from the Délégation Générale à la Recherche Scientifique et Technique, the Commissariat à l'Energie Atomique, France and the Fondation pour la Recherche Médicale Française.

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