Morphological Stages of *Bacillus subtilis* Sporulation and Resistance to Fusidic Acid

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During spore development of *Bacillus subtilis* both protein synthesis and sporulation become resistant to the antibiotic fusidic acid. This resistance develops at the time when asymmetric prespore septa are formed. Simultaneously ribosomes lose their ability to bind fusidic acid, as demonstrated by their affinity chromatography with the immobilized drug. Mutants resistant to fusidic acid during growth are oligosporogenous; their sporulation development is blocked before septum formation. These results indicate that normal ribosomes are needed for prespore septation sporulation; only after septation can protein synthesis be maintained, throughout the development period, by fusidate resistant ribosomes.

INTRODUCTION

Massive sporulation of bacilli occurs after they have stopped growing in sporulation media. Subsequently, many metabolic changes occur which cause the transcription and translation of genes, some of which are specifically required for sporulation. Translation of the mRNA formed throughout the developmental period is accompanied by extensive RNA and protein turnover (for review, see Kornberg *et al.*, 1968). The translation machinery for *de novo* protein synthesis originates in vegetative cells, although the continuing synthesis of ribosomal RNA suggests that new ribosomes are formed during the sporulation process (for review, see Cundliffe, 1972). Some translational functions are altered during spore formation of *Bacillus subtilis*. Whereas the ribosomal functions mediated by G-factor (EF-G) are sensitive to inhibition by the antibiotic fusidic acid in vegetative cells, they become resistant during spore formation (Fortnagel & Bergmann, 1973; Fortnagel, 1973; Fortnagel *et al.*, 1975). *In vitro* the drug inhibits the GTPase activity of 70S ribosome-EF-G complexes isolated from growing cells, but does not inhibit those from sporulating cells. Apparently, normal ribosomal functions, including those identified by their sensitivity to fusidic acid, are required for the initiation of normal sporulation, because mutants already resistant to fusidic acid during growth are oligosporogenous (Fortnagel, 1973).

We report here the developmental stage at which the standard strain of *B. subtilis* becomes resistant to fusidic acid and at which stage fusidic acid resistant mutants are blocked in their development.

METHODS

Bacterial strains. Derivatives of the transformable Marburg strain of *Bacillus subtilis* were used throughout. Our standard strain (60015) required tryptophan (indole) and methionine for growth (Freese & Fortnagel, 1967). To isolate spontaneous fusidic acid resistant mutants, strain 60015 was grown in nutrient sporulation medium (NSM; Schaeffer *et al.*, 1965) from a single colony. Approximately 10^8 bacteria ml⁻¹ were

inoculated onto plates containing 33 g Difco tryptose blood agar base l⁻¹ (TBAB plates). Sodium fusidate (50 µl of 1 mM filter-sterilized solution) was pipetted into a well stamped into the agar of each plate. After 3 days incubation at 37 °C small colonies developed in the inhibition zone. Only one resistant mutant was picked from each plate to ensure that distinct mutants were isolated. Sporulating revertants of these mutants were obtained after treatment with 1 µg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine ml⁻¹ in 0.25 M-phosphate buffer pH 6.5 for 30 min at 37 °C (Freese & Fortnagel, 1967). The treated samples were diluted into NSM, grown overnight at 37 °C, heated for 15 min at 75 °C to inactivate vegetative cells, and plated on TBAB plates. Revertants were detected by their brown colony appearance after 3 days at 37 °C compared with the pale colonies of fusidic acid resistant mutants. To obtain distinct revertants, only one revertant colony was isolated from each culture.

Growth conditions. Bacteria were grown in NSM at 37 °C in Erlenmeyer flasks in a reciprocal water-bath operating at 120 strokes min⁻¹. The flasks were filled to not more than one-fifth of their volume to allow optimum aeration. Growth was followed by measuring the absorbance of the cultures in an Eppendorf photometer equipped with a monochromatic filter of 578 nm. Cell titres were determined after dilution in 0.1 M-phosphate buffer pH 6.5, containing 1 mM-MgCl₂, and plating on TBAB plates. Spore titres were determined after heating the dilutions for 15 min at 75 °C. The notation *T_n*, where *n* is the time in hours after exponential growth ceased, is used to indicate the sporulation stage of the culture.

Fusidic acid inhibition. Minimum inhibitory concentrations (m.i.c.) were determined by the agar diffusion method (Sherwood, Falco & de Beer, 1944). Growth and inhibition of sporulation were measured in NSM.

Amino acid incorporation. At different growth stages in NSM 0.5 ml culture samples were transferred to test tubes (20 mm diam.) containing 0.1 µCi uniformly ¹⁴C-labelled protein hydrolysate (57 mCi matom⁻¹) which gave a final concentration of 0.3 × 10⁻⁸ M; fusidic acid or chloramphenicol were present at final concentrations of 5 × 10⁻⁸ M or 2.5 × 10⁻⁴ M respectively. The samples were shaken at 37 °C for 30 min; 1 ml ice-cold 10 % (w/v) trichloroacetic acid was added and, after standing for 30 min in ice, the samples were collected on membrane filters (Sartorius, type 11106, 0.45 µm) and washed five times with approximately 5 ml ice-cold 5 % trichloroacetic acid. The filters were dissolved in 10 ml Bray's solution (Bray, 1960) and the radioactivity was determined in a Beckman 230 scintillation spectrometer.

Affinity binding of ribosomes to immobilized fusidic acid. Fusidic acid was coupled to Sepharose 6B via the spacer 1,4-butanediol-diglycidyl ether (Porath, 1974). A column (0.5 × 2.5 cm) was filled with the immobilized drug and equilibrated with buffer containing: 10 mM-Tris/HCl, 10 mM-magnesium acetate, 50 mM-NH₄Cl, 6 mM-2-mercaptoethanol and 1 mM-GTP, pH 7.8. The total capacity of such a column was approximately 5 mg ribosomes per ml packed gel.

Bacteria were grown in NSM in the presence of [2-¹⁴C]uracil (0.15 µCi ml⁻¹; 10⁻⁴ M) for three generations. At different times of growth and sporulation, samples were taken and harvested at 0 °C by centrifuging for 5 min at 48000 g. Bacteria were washed twice in the above buffer; after resuspension in this buffer they were lysed in a French press and then centrifuged for 5 min at 48000 g. The supernatant fraction was used as the crude cell extract. This extract, containing less than one-tenth of the total column capacity for ribosomes, was immediately added to the fusidate column at 25 °C to allow optimum complex formation. After 1 min the temperature was lowered and maintained at 0 °C. The column was washed with the above buffer lacking GTP until no further radioactive or ultraviolet light-adsorbing material was eluted. This washing procedure was completed in less than 5 min. Thereafter ribosomes were eluted with buffer containing 250 mM-NH₄Cl. The fraction of the total [¹⁴C]uracil label in this eluate is a direct measure of the complex-forming capacity of ribosomes with fusidic acid in the presence of GTP (Fortnagel, 1977).

Electron microscopy. In order to determine the morphological stage at which fusidic acid resistance occurred, samples were taken from the original culture at different times during growth and the developmental period. They were fixed with glutaraldehyde, post-fixed with osmium tetroxide, and stained with uranyl acetate and lead citrate as described previously (Freese, Cooney & Freese, 1975). For each sample, 200 to 300 longitudinally sectioned cells were analysed with a Philips 201 electron microscope. The results were statistically evaluated as described by Freese *et al.* (1975). The engulfment substages were defined (see Fig. 3) so that they agree with the original definitions used for mutants by Ryter, Schaeffer & Ionesco (1966) and the recent review of Piggott & Coote (1976), rather than the definitions used by others.

RESULTS

Influence of fusidic acid on growth and sporulation

Growth of the standard strain *B. subtilis* 60015 in nutrient sporulation medium was inhibited by fusidic acid concentrations of 50 nM or higher. Similarly, the formation of heat-resistant spores was inhibited if fusidic acid was added during exponential growth or within

Fig. 1. Influence of fusidic acid on growth and sporulation of *Bacillus subtilis*. The standard strain 60015 was grown in NSM. Growth was followed by measuring E_{578} (Δ). At different times, 1 ml samples were transferred to test tubes containing 5 nmol fusidic acid. The tubes were shaken at 37 °C and the titres of both total colony forming units (V = vegetative cells plus spores; $\circ$) and heat-resistant colony forming units (S = spores; $\bullet$) were determined 20 h after the beginning of the experiment.

the first hour after exponential growth ceased, i.e. up to T_1 (Fig. 1). Subsequently, fusidic acid sensitivity decreased; spores were produced at the normal frequency within the next 10 h even in the presence of 5 μ M-fusidate, provided that this was added after T_2 .

The rate of incorporation of a ^{14}C -labelled amino acid mixture into acid-precipitable material decreased rapidly after T_0 in the untreated control culture (Fig. 2a); it reached a minimum value at $T_{0.5}$ and remained low during the following sporulation period. Both fusidic acid and chloramphenicol inhibited amino acid incorporation during growth. The chloramphenicol inhibition continued beyond T_1 , whereas the incorporation in the presence of fusidic acid increased after T_1 and reached the level of incorporation of the untreated culture at $T_{2.5}$; thus protein synthesis was then completely resistant to a 100-fold excess of fusidate. The appearance of sporulation resistance paralleled the increasing resistance of the amino acid incorporation (Fig. 2b).

That the activity of fusidic acid did not decrease during this prolonged treatment was shown by the following experiment. The standard strain (60015) was grown in NSM to T_2 and then fusidate was added to a final concentration of 1 μ M. Immediately, and at intervals up to 24 h, samples were taken and the biological inhibitory activity of the drug-containing solution was measured against exponentially growing *B. subtilis* by the agar diffusion method. No inactivation of the drug could be demonstrated. This method could detect a 10% inactivation, as was shown by dilution of the original fusidate-containing culture.

Affinity binding of ribosomes to immobilized fusidic acid

Fusidic acid forms a stable complex with 70S ribosomes (EF-G and GDP) even when the drug is covalently bound to Sepharose 6B via the spacer molecule 1,4-butanediol-diglycidyl ether (Fortnagel, 1977). Thus ribosomes from the extract of sensitive vegetative *B. subtilis* were quantitatively retained by fusidate columns in the presence of 1 mM-GTP and 50 mM- NH_4Cl . They could be eluted with 250 mM- NH_4Cl . Binding did not occur when cell extracts of fusidate resistant mutants (Table 1) were used.

Fig. 2. (a) Rate of incorporation of ^{14}C -labelled amino acid into acid-precipitable material during growth and sporulation. Strain 60015 was grown in NSM. At different times samples were taken and incubated for 30 min with ^{14}C -labelled amino acids (protein hydrolysate) alone (●) or with added 5×10^{-6} M-fusidic acid (△) or 2.5×10^{-4} M-chloramphenicol (▲). The acid-precipitable radioactivity (c.p.m.) was determined and plotted per E_{578} unit of the culture [c.p.m. (E_{578} unit) $^{-1}$]. Growth was followed by measuring E_{578} (○).

(b) Relative rate of incorporation of ^{14}C -labelled amino acid. The values are expressed as the ratio of the incorporation in the presence of either fusidic acid (△) or chloramphenicol (▲) to the incorporation without inhibitors. Spore formation in the presence of fusidic acid was measured as in Fig. 1; the sporulation frequency (S/V; ●) is equal to the titre of heat-resistant colony forming units divided by the titre of total colony forming units.

If the development of resistance of sporulation and of amino acid incorporation into protein after T_2 occurred as a result of a change in the site of the ribosome-EF-G complexes to which fusidate adsorbs, one would expect that binding of the drug would disappear during sporulation. This is actually the case (Fig. 3). During growth and up to T_1 , the affinity binding of [2- ^{14}C]uracil-labelled material reached 80 % of the total amount applied. Thereafter, the bound fraction decreased; at T_2 , less than 20 % of the label was bound and this low binding remained constant for later times. This residual binding may represent unmodified ribosomes. Non-specific adsorption to the modified Sepharose can be ruled out; no binding was found in the presence of 50 mM- NH_4^+ with deoxycholic acid bound via the above spacer to Sepharose 6B. The loss of binding capacity coincided with the development of resistance during sporulation (Fig. 3).

Correlation of fusidic acid resistance and morphological stages of development

To determine whether the development of resistance to fusidate corresponded to a certain morphological stage of development, both properties were measured in the same culture (Fig. 3). The sporulation stages are defined in the legend to Fig. 3. An excellent agreement was found between resistance of sporulation and stage IIa^+ of development (IIa^+ indicates that cells have reached stage IIa or any later stage); apparently cells became resistant to fusidate as soon as they had formed the prespore septum. The midpoints (50 %) of the curves for the development of sporulation resistance and stage IIa^+ both occurred at $T_{1.3}$; they coincided with the midpoints of the curves for the increase in resistance of amino acid incorporation (Fig. 2b) and the decrease in binding of [^{14}C]uracil-labelled

Fig. 3. Comparison of development of fusidate resistance with morphological stages of sporulation. ●, Sporulation frequency (S/V) in the presence of fusidic acid, determined as in Fig. 2(b). □, Percentage of RNA ($[^{14}\text{C}]$ uracil-labelled material) bound to immobilized fusidic acid in the presence of 50 mM- NH_4^+ . Sporulation stages are defined as: 0-I, cells without prespore septa (▲); IIa, cells with a developing up to a finished asymmetric septum (△); IIb, cells with prespore membrane curved up to a 50 % engulfment (▽); IIc, cells with 50 % prespore membrane engulfment up to almost complete enclosure of the prespore (▼); III, cells in which the prespore is completely engulfed in the double membrane (○). + signifies inclusion of all cells at later stages of development, e.g. IIa+ includes all cells with a developing asymmetric septum or at a later morphological stage.

material to immobilized fusidic acid (Fig. 3). When fusidate was added before $T_{0.5}$ no prespore septa developed and no sporulation took place. The minimum inhibitory concentration of 50 nM-fusidic acid then led to the formation of long cell filaments.

Fusidic acid resistant mutants

Two hundred distinct spontaneous fusidate resistant mutants (gene symbol *fus*) were isolated. These mutants were oligosporogenous as is shown for five representatives in Table 1. They grew at the normal rate and to the normal cell titre as judged by the absorbance of the cultures. Thus their growth properties were indistinguishable from the sporulating standard strain. Revertants with normal ability to sporulate were isolated from the individual *fus* mutants after nitrosoguanidine treatment. These revertants were all sensitive to fusidic acid.

When the five *fus* mutants of Table 1 were examined with an electron microscope at T_5 , 99 % of the cells had not reached stage IIa of development, i.e. no prespore septa were visible. In contrast 78 % of the cells from the control culture of the standard strain 60015 had proceeded at least to stage IIa and mostly beyond. Although the mutants did not sporulate, wall synthesis must have continued at least for a while, since the mutant cells at T_5 had much thicker walls than exponentially growing cells or sporulating *B. subtilis* cells at T_5 .

That the *fus* mutants did not destroy the added fusidate was shown by the agar diffusion method described for the standard strain above.

Table 1. *Growth and sporulation properties of fusidic acid resistant mutants of B. subtilis*

Strain	Parent strain	Isolated phenotype	Treatment	M.i.c. of fusidic acid (M)	Total titre after 24 h in NSM (c.f.u. ml ⁻¹)	Frequency of spores after 24 h in NSM
60015	standard strain			5×10^{-8}	2.8×10^8	8.8×10^{-1}
70044	60015	Fus	spontaneous	6×10^{-4}	4.7×10^8	1.2×10^{-4}
44r ₇	70044	Spo ⁺	NTG	4×10^{-7}		5.7×10^{-1}
44r ₅	70044	Spo ⁺	NTG	5×10^{-8}		6.0×10^{-1}
70045	60015	Fus	spontaneous	9×10^{-5}	2.3×10^8	6.5×10^{-4}
45r ₁	70045	Spo ⁺	NTG	5×10^{-8}		7.0×10^{-1}
45r ₂	70045	Spo ⁺	NTG	4×10^{-8}		7.2×10^{-1}
45r ₃	70045	Spo ⁺	NTG	1×10^{-8}		4.5×10^{-1}
70046	60015	Fus	spontaneous	2×10^{-5}	3.0×10^8	9.2×10^{-3}
70047	60015	Fus	spontaneous	2×10^{-5}	2.9×10^8	1.5×10^{-3}
47r ₁	70047	Spo ⁺	NTG	1×10^{-7}		7.0×10^{-3}
47r ₂	70047	Spo ⁺	NTG	5×10^{-8}		4.2×10^{-3}
70048	60015	Fus	spontaneous	8×10^{-5}	1.5×10^8	2.3×10^{-3}
48r ₁	70048	Spo ⁺	NTG	3×10^{-7}		9.2×10^{-1}
48r ₂	70048	Spo ⁺	NTG	7×10^{-8}		7.2×10^{-1}
48r ₃	70048	Spo ⁺	NTG	1×10^{-7}		1.0×10^{-1}
48r ₄	70048	Spo ⁺	NTG	3×10^{-7}		4.4×10^{-1}
48r ₅	70048	Spo ⁺	NTG	5×10^{-8}		6.5×10^{-1}

NTG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

DISCUSSION

The ability of the standard strain of *Bacillus subtilis* to form spores in nutrient sporulation medium changes from being sensitive to fusidate to being resistant at about $T_{1.5}$, at the same time as the asymmetric prespore septum is formed. In contrast, sporulation remains sensitive to chloramphenicol and other antibiotics (Sterlini & Mandelstam, 1969) throughout development.

The observed transition could arise from several cellular changes. (i) A modification of the cell membrane composition or structure might exclude fusidate from further entry or transport into the cells. Such a change was, for example, found in fusidate resistant mutants of *Staphylococcus aureus* (Chopra, 1976). Since we did not have radioactively labelled fusidic acid, this possibility could not be examined here. (ii) Fusidate could be enzymically modified or degraded and thus inactivated. Our measurements, which allow detection of 10 % degradation, have excluded this possibility. (iii) Resistance could occur by a change of the protein synthetic machinery, such as an alteration of ribosomes or of the translocation factor EF-G which is a known site of fusidate attack (Cundliffe, 1972).

The GTPase activity of purified ribosomes can be measured uncoupled from protein synthesis; it depends on the addition of EF-G. Irrespective of the source of EF-G, this reaction is sensitive to fusidate if the ribosomes are isolated from vegetative cells of the standard strain, but it is resistant if the ribosomes are isolated from sporulating cells after T_2 (Fortnagel, 1977). The GTPase activity is also resistant to fusidate if EF-G-ribosomal complexes are isolated from vegetative cells of fusidate resistant mutants (Fortnagel & Bergmann, 1973).

In the presence of GTP, the affinity binding of crude ribosomes containing the translocation factor EF-G to immobilized fusidic acid greatly decreases between $T_{0.5}$ and $T_{1.5}$ (Fortnagel, 1977); at the same time, both the EF-G dependent GTPase activity (*in vitro*) and the amino acid incorporation decline. These events clearly demonstrate a ribosomal change early in sporulation. It remains an intriguing possibility that this change may be actually necessary for sporulation.

Formation of the asymmetric prespore septum and the development of resistance to fusidate occur simultaneously. Apparently, normal ribosomes are needed for prespore septation. Thereafter, the altered ribosomes suffice (or are needed?) to maintain protein synthesis during the continuing development. This conclusion agrees with the finding that *fus* mutants, which are already resistant to fusidate during exponential growth, are blocked in their sporulation development before the prespore septum is formed. But it is not known whether the asymmetric septation and the ribosomal changes are causally related or are only by chance correlated in time. The results certainly show that the development of fusidate resistance does not depend on the engulfment of the forespore; it occurs earlier.

Although these findings clearly demonstrate a change of ribosomes early in sporulation, we cannot yet exclude the possibility that other changes might occur which influence fusidate sensitivity of *B. subtilis*. For example, a modification of the cell membrane composition or structure might block the further uptake of fusidate during sporulation.

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