Effect of Antibiotics on Sporulation Caused by the Stringent Response in *Bacillus subtilis*

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We have examined in *Bacillus subtilis* how antibiotics that inhibit protein or RNA synthesis affect the stringent response (i.e. accumulation of (p)ppGpp) and the resulting decrease of GTP and sporulation. All antibiotics used inhibited sporulation completely at concentrations at which they inhibited growth only partially and in most cases only slightly. At these concentrations, some antibiotics (chloramphenicol, fusidic acid, lincomycin, tetracycline) abolished the stringent control, others lowered it. Sporulation inhibited by chloramphenicol or fusidic acid could be almost completely restored by addition of 0.2–0.5 mM-decoyinine, an inhibitor of GMP synthetase. The inhibition of sporulation by tetracycline and streptomycin, or by the RNA polymerase inhibitors rifampin and lipiarmycin, was partially counteracted by decoyinine, whereas the inhibition by lincomycin or by compounds that did not interfere with the stringent response could not be overcome by decoyinine addition. In mutants resistant to erythromycin, or lincomycin or thiostrepton, sporulation was no longer inhibited by that particular antibiotic but was still sensitive to the other two.

The results show that some antibiotics inhibited sporulation because they prevented the decrease of GTP needed to initiate sporulation caused by the stringent response. Other antibiotics inhibited sporulation by interfering with specific (ribosomal) functions either needed to maintain a certain metabolic state or specifically required for the synthesis of sporulation-specific proteins.

INTRODUCTION

Numerous experimental conditions used to initiate sporulation of *Bacillus subtilis* cause a partial deprivation of intracellular amino acids and thus produce the stringent response including the accumulation of ppGpp and pppGpp (the combination of both compounds will be indicated by (p)ppGpp). Relaxed (*relA*) mutants, unable to produce the stringent response (Gallant, 1979), do not sporulate under these conditions (Freese, 1981; Lopez *et al.*, 1981a; Ochi *et al.*, 1981). The stringent response also causes the inhibition of IMP dehydrogenase and therefore a decrease in the intracellular concentration of the major guanine nucleotides, including GTP (Lopez *et al.*, 1981a). Both stringent and relaxed strains can be induced to sporulate by inhibitors of GMP (and thus GTP) synthesis, such as mycophenolic acid (which inhibits IMP dehydrogenase) or decoyinine (which inhibits GMP synthetase), or by guanine deprivation of a relaxed guanine auxotroph. These results indicated that the initiation of sporulation resulted not from the accumulation of (p)ppGpp but from the decrease of GTP (or GDP) (Lopez *et al.*, 1979, 1981a). This was confirmed with a mycophenolate-resistant *ilv* auxotroph. Upon partial isoleucine deprivation this mutant still produced the high amounts of

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(p)ppGpp but no longer the pronounced GTP decrease, and it no longer sporulated. Nevertheless, its sporulation could still be induced by decoyinine, which caused a further GTP decrease (Ochi *et al.*, 1982).

Several groups have found that various antibiotics binding to ribosomes inhibit sporulation when used at concentrations at which they have little effect on growth (see Freese, 1981). This could imply that particular ribosomal properties, affected by the antibiotics, are more important for sporulation than for growth. But because those experiments were performed in media in which sporulation resulted from the stringent response, it was also possible that the antibiotics prevented sporulation simply by reducing the extent of this response. In fact, the inhibition of sporulation by fusidic acid or kasugamycin could be counteracted by decoyinine (Lopez *et al.*, 1981 *b*). Therefore, we have here examined which of the commonly used antibiotics that prevent sporulation caused by the stringent response do so by interfering with this response. For this purpose, we have used decoyinine, which decreases GTP without any accumulation of (p)ppGpp, to determine whether it can restore sporulation. We show that most antibiotics inhibiting protein or RNA synthesis are much more potent inhibitors of sporulation than of growth. At low inhibitor concentrations, just sufficient to prevent sporulation caused by the stringent response, some of the antibiotics prevent the accumulation of (p)ppGpp and the concomitant decrease of GTP; for some of these compounds, sporulation can be restored by addition of decoyinine. The results enable the antibiotics to be sub-divided according to their effects on the stringent response and on differentiation.

METHODS

Bacterial strains and growth conditions. All experiments except those in Table 2 were performed with *B. subtilis* strain 61886 (*de-iloB1 kauA1*; 'de-' stands for deletion) which was grown in synthetic medium (Ochi *et al.*, 1981) containing 1% (w/v) glucose and 0.5 mM of both L-isoleucine (Ile) and L-valine. When the A_{600} was 0.5, the cells were rapidly washed on a membrane filter and transferred to the same medium (Ile-medium) or to the equivalent medium which contained 0.4 mM-DL-oxomethylvalerate instead of isoleucine (Omv-medium). Each culture was distributed into flasks containing different amounts of various antibiotics and decoyinine at stated concentrations. The antibiotics were dissolved in water or ethanol [final concentration of ethanol was less than 0.5% (v/v)]. Thirteen hours after cell transfer, the titre of viable cells was determined by diluting the culture in 0.1 M-potassium phosphate buffer, pH 7, containing 1 mM-MgCl₂ and plating on tryptose blood agar base (TBAB; Difco) plates. To determine the spore titre, the dilution tubes were heated for 20 min at 75 °C and then also plated on TBAB plates.

Strain 61885 (*de-iloB1 kauA1 relA1*) was isogenic with strain 61886 with the exception of the *relA* marker (Ochi *et al.*, 1981). Strain 62236 (*lin-2 metC3 trpC2*) was derived by transformation of strain 61653 (*metC3 trpC2*) with DNA of strain 61551 (*lin-2* strain Lin + UVB29 of Dubnau) and selection for colonies resistant to 40 µg lincomycin ml⁻¹ on TBAB plates. Strain 62100 (*ery-I*, prototroph) was provided by Dr T. Leighton and strain 61953 (*thr-5 trpC2 relC* where *relC* = *tsp-6*) by Dr I. Smith.

All strains except 61953 grew well in synthetic medium containing 0.5 mM of the stated required compounds (doubling times 45–60 min). Strain 61953 grew very slowly in synthetic medium plus threonine and tryptophan (doubling time more than 4 h) but grew at the same rate as the other strains if the medium was supplemented with Casamino acids (vitamin-free, 0.3%, w/v; Difco). We do not know whether the slow growth without Casamino acids indicated a leaky deficiency for another amino acid or resulted from the rather perfect *rel* property of the *relC* mutation. After tryptophan deprivation, the concentration of ppGpp and pppGpp remained less than 1 pmol per absorbance unit (A_{600}), whereas the *relA* strain 61885, which grew in Ile-medium with a doubling time of 60 min, produced 6 pmol ppGpp per absorbance unit after isoleucine deprivation. A basal level of ppGpp may be essential to maintain growth at the normal rate in synthetic medium in which the cells have to synthesize most amino acids. Growth of the *ery*, *lin*, and *relC* strains used here was resistant to at least 4 µg erythromycin ml⁻¹, 100 µg lincomycin ml⁻¹ or 10 µg thiostrepton ml⁻¹ if the cells were grown in synthetic medium (containing 0.3%, w/v, vitamin-free Casamino acids in the case of strain 61953). Each of the three strains was sensitive to the other two antibiotics. For comparison, the concentrations inhibiting growth of strain 61886 by 50% are given in Table 1. The *relA* strain 61885 and strain 61653 were also sensitive to all three antibiotics.

Nucleotide measurements. To determine the concentration of intracellular nucleotides, cells from 100 ml culture were collected within 20 s on a membrane filter (pore size 0.45 µm, diameter 10 cm; Schleicher and Schuell), and the filter was immediately laid upside down on to 1.5 ml ice-cold formic acid (0.5 M) in a plastic Petri dish. After 60 min at 4 °C, the filter and the cells were removed by centrifugation and the supernatant was filtered through a syringe fitted with a membrane filter. After lyophilization, the residue was dissolved in 130 µl deionized water;

60 µl of the solution was applied to a column of partisil PXS 10/25 SAX (Whatman), SO_4^{2-} form and chromatographed by HPLC as described (Ochi *et al.*, 1981).

We have checked different concentrations of formic acid for the extraction of (p)ppGpp and ATP and found no difference in the extraction efficiency in the range of 0.5 M to 2 M-formic acid. Therefore we used the lower concentration. During lyophilization, the concentration of formic acid increases because water is more easily evaporated first. Because nucleotides are labile in strong acid, it was essential to use a rapid pump that maintained a vacuum good enough to keep the material frozen throughout evaporation and to remove the formic acid rapidly.

Source of drugs. Actinomycin D, chloramphenicol, erythromycin, puromycin dihydrochloride, rifampin and streptomycin sulphate were purchased from Sigma, and lincomycin. HCl and tetracycline. HCl from Upjohn Co., Kalamazoo, Mich., U.S.A. The following compounds were received as gifts: decoyinine from Dr J. E. Grady, Upjohn Co., Kalamazoo, Mich., U.S.A.; fusidic acid from Dr P. Fortnagel, University of Hamburg, Germany; lipiarmycin from Dr G. Lancini, Gruppo Lepetit, Milan, Italy; and thiostrepton from Dr S. J. Lucania, E. R. Squibb & Sons, Inc., Princeton, N. J., U.S.A.

RESULTS

Inhibition of growth

To determine the growth inhibitory potency of the different antibiotics we grew strain 61886 in Ile-medium to $A_{600} = 0.5$ and then transferred the culture to flasks containing different amounts of an antibiotic. With most antibiotics, the cultures continued to grow exponentially but the growth rate (measured by plotting $\log A_{600}$ against time) decreased with increasing antibiotic concentrations. However, some antibiotics (lincomycin, lipiarmycin, rifampin and tetracycline) inhibited growth more intensely immediately after their addition, whereas puromycin inhibited maximally only after some time. While these phenomena may be important for the explanation of the detailed molecular effect of each antibiotic, they are probably not essential to explain the results presented here. To allow for these differences in the time of maximum response, we determined the doubling times over the time ranges stated in Table 1. The ratio of these values in the presence and absence of antibiotic was taken as a measure of inhibition; by interpolation between values obtained for different concentrations of antibiotic, we determined the concentration causing 50% growth inhibition (Table 1). This concentration ranged from 0.01 µg ml⁻¹ (for rifampin) to 55 µg ml⁻¹ (for spectinomycin).

Table 1. *Potency of antibiotics to inhibit growth of B. subtilis*

Strain 61886 was grown in Ile-medium at 37 °C. When the A_{600} was 0.2, the culture was distributed to flasks containing different amounts of the antibiotic and the shaking of the flasks was continued for 6 h. The concentration causing 50% growth inhibition was determined by interpolation of rates obtained at different concentrations.

Antibiotic	Time range in which inhibition was measured* (h)	Concentration causing 50% growth inhibition (µg ml ⁻¹)
Actinomycin	0-3	0.09
D(-)-threo-Chloramphenicol	0-3	2.3
Erythromycin	0-3	0.25
Fusidic acid	0-3	0.20
Lincomycin	0-1	5.5
Lipiarmycin	0-1	0.38
Puromycin	2-4	6.0
Rifampin	0-1	0.008
Spectinomycin	0-3	55
Streptomycin	0-3	48
Tetracycline	0-3	1.2
Thiostrepton	0-3	1.0

* Lipiarmycin and rifampin inhibited growth strongly for only 1 h, lincomycin and tetracycline for only 3 h, whereas puromycin inhibited growth significantly for only 2 h after their addition. The other antibiotics inhibited growth to the same degree throughout the 6 h range.

Fig. 1. Effects of antibiotics on sporulation initiated by partial Ile deprivation or by decoyinine addition. Strain 61886 was grown at 37 °C in Ile-medium to $A_{600} = 0.5$. The cells were washed on a membrane filter and transferred to Ile-medium plus decoyinine or to Omv-medium plus or minus decoyinine at the following concentrations: (a) 0.3 mM; (b) 0.6 mM; (c-h) 0.3 mM. Ten ml of each cell suspension were distributed into different 125 ml flasks containing various amounts of each antibiotic tested. The cultures were shaken at 37 °C and the heat-resistant spore titre was measured 13 h later. ●, Omv-medium plus antibiotic; ○, Omv-medium plus antibiotic plus decoyinine; △, Ile-medium plus antibiotic plus decoyinine. The arrows designate the concentration (in µg ml⁻¹) at which the antibiotic caused 50% growth inhibition in Ile-medium; for chloramphenicol and thiostrepton the value is written next to the arrow because it is off scale.

Inhibition of sporulation

To initiate sporulation, we grew strain 61886 in Ile-medium to $A_{600} = 0.5$, washed the cells with synthetic medium and suspended them in a medium (Omv-medium) containing a growth-limiting amount of oxomethylvalerate (in place of Ile) and the stated amount of an antibiotic. We measured sporulation 13 h later; this time allowed complete spore maturation (to heat resistance) even when an antibiotic and decoyinine were added. As observed for the inhibition of growth, the concentrations of different antibiotics needed to reduce sporulation by a factor of 1000 or more differed vastly, rifampin being the most, and streptomycin the least, potent inhibitor (Fig. 1).

As would be expected from their inhibition of RNA or protein synthesis, all antibiotics inhibited sporulation. But remarkably, most compounds inhibited sporulation severely at concentrations at which they inhibited growth only minimally (Fig. 1; for ease of comparison the 50% inhibitory concentration in Table 1 is shown by an arrow). This was particularly

pronounced for chloramphenicol (Fig. 1*b*) and spectinomycin (data not shown; with $15 \mu\text{g ml}^{-1}$ the spore titre was 30 ml^{-1}), which prevented sporulation at concentrations that were three times lower than those needed to inhibit growth by 50%. For puromycin, $8 \mu\text{g ml}^{-1}$ prevented sporulation (spore titre $< 10 \text{ ml}^{-1}$; data not shown). Streptomycin (Fig. 1*f*) was the only compound for which the concentration needed to inhibit sporulation strongly was higher than that needed to inhibit growth by 50%.

For some compounds the inhibition curve showed a threshold, sporulation being affected very little or not at all at low concentrations, followed by a steep decline of sporulation at higher concentrations. This was observed for erythromycin (Fig. 1*e*), streptomycin (Fig. 1*f*) and for actinomycin (data not shown) which inhibited sporulation very little up to $0.015 \mu\text{g ml}^{-1}$ ($0.04 \mu\text{g ml}^{-1}$ prevented sporulation: spore titre $< 10 \text{ ml}^{-1}$).

Effect of decoyinine on the inhibition of sporulation

The optimal concentration of decoyinine inducing maximal sporulation in Ile-medium was 0.2 mM. In the presence of an antibiotic the optimal decoyinine concentration might be different. Therefore, we first measured the effect of different decoyinine concentrations on sporulation produced after cell transfer to Omv-medium containing an amount of antibiotic that reduced the spore titre to less than $2 \times 10^3 \text{ ml}^{-1}$ (except for rifampin where the titre was 10^4 ml^{-1}). We found (data not shown) optimal sporulation at a decoyinine concentration of 0.3 mM for the following antibiotics ($\mu\text{g ml}^{-1}$): fusidate (0.14), lipiarmycin (0.5), rifampin (0.03), streptomycin (130) and tetracycline (3) and at 0.6 mM-decoyinine for chloramphenicol (0.7). No significant increase in the spore titre was observed at any decoyinine concentration for erythromycin (0.2), lincomycin (10), puromycin (6), spectinomycin (20) or thiostrepton (0.6).

We then examined how much sporulation could be induced by decoyinine in the presence of different concentrations of each antibiotic. We used the decoyinine concentration which had been found above to be optimal for sporulation with a given antibiotic (if the sporulation frequency could not be increased by any concentration of decoyinine, the results with 0.3 mM-decoyinine are reported). Two conditions were studied: (1) Cells were transferred, as above, to Omv-medium containing the stated amount of antibiotic and the optimal concentration of decoyinine. (2) Cells were left in Ile-medium to which different amounts of an antibiotic and decoyinine (at the optimal concentration) were added (at $A_{600} = 0.5$). In the latter medium, cells did not experience an amino acid shift-down and did not produce (p)ppGpp; they sporulated solely due to the presence of decoyinine. Such induction of sporulation by decoyinine is more efficient than that caused by deprivation of a single amino acid (Ochi *et al.*, 1981): it measures the extent to which cells are still capable of sporulating in the presence of an antibiotic.

The sporulation response to decoyinine addition differed vastly with different antibiotics (Fig. 1). The inhibitory effects of fusidate (Fig. 1*a*) and chloramphenicol (Fig. 1*b*) were almost completely prevented by decoyinine addition. Only at much higher concentrations did these antibiotics significantly inhibit sporulation even in the presence of decoyinine (not shown). The inhibitory effect of tetracycline (Fig. 1*c*) and thiostrepton (Fig. 1*d*) could be partially counteracted by decoyinine. The effect of erythromycin in Omv-medium was not significantly counteracted by decoyinine; in Ile-medium + decoyinine, erythromycin also inhibited sporulation but slightly less potently than in Omv-medium (Fig. 1*e*). The inhibition observed when the concentration of actinomycin (data not shown) or streptomycin (Fig. 1*f*) was above the threshold could be only partially counteracted by decoyinine and the magnitude of this recovery was variable (with $0.04 \mu\text{g actinomycin ml}^{-1}$ and 0.3 mM-decoyinine the spore titre was 500 ml^{-1} ; in Ile-medium plus decoyinine the spore titre was tenfold higher). At all concentrations, lincomycin (Fig. 1*g*) as well as puromycin and spectinomycin (data not shown) inhibited sporulation to the same degree with and without decoyinine.

The two RNA polymerase inhibitors, rifampin (Fig. 1*h*) and lipiarmycin (data not shown), apparently inhibited sporulation by two effects. One reaction occurred at very low antibiotic concentrations and could be completely counteracted by decoyinine; the other reaction was significant only at higher antibiotic concentrations. With rifampin, the inhibition curve showed a sudden bend at a concentration of about $0.01 \mu\text{g ml}^{-1}$, at which about 10^4 spores were

Fig. 2. Changes in the intracellular concentrations of ppGpp (a, b) and GTP (c, d) after cell transfer to Omv-medium containing certain antibiotics plus or minus decoyinine. Strain 61886 was grown in several flasks, each containing 100 ml Ile-medium, to $A_{600} = 0.5$. The cells were washed and suspended in 100 ml Omv-medium which contained the antibiotic at a concentration that almost completely prevented sporulation (see Fig. 1); some of the flasks also contained decoyinine (for concentration see Fig. 1). At indicated times, the cells were collected on a membrane filter, and extracted with 0.5 M-formic acid; the nucleotide composition of the extract was analysed by HPLC. (a, c) Omv-medium: alone (●). (b, d) Omv-medium: plus 1 μg chloramphenicol ml^{-1} (▲); plus 1 μg chloramphenicol ml^{-1} plus 0.6 mM-decoyinine (▽); plus 0.14 μg fusidic acid ml^{-1} (Δ); plus 0.14 μg fusidic acid ml^{-1} plus 0.3 mM-decoyinine (■).

produced per ml. This is approximately the spore titre that would be expected in a culture not exposed to amino acid deprivation. Thus, very low concentrations of rifampin apparently prevented the sporulation caused by the stringent response but not that produced spontaneously. At these low rifampin concentrations, decoyinine addition permitted efficient sporulation. With increasing lipiarmycin concentrations, the spore titre initially decreased more slowly (presumably because lipiarmycin does not bind to RNA polymerase as tightly as does rifampin) so that the transition to the second, shallower decline (which started at $0.2 \mu\text{g ml}^{-1}$) was smooth. Whereas the initial decrease could be counteracted by decoyinine, the slope of the second decline was essentially the same in the absence and the presence of decoyinine (in Omv- or Ile-medium).

Nucleotide measurements

To determine which antibiotics may have prevented sporulation by reducing the stringent response, we measured the intracellular concentration of ppGpp at different times after the cells had been transferred to Omv-medium which contained the antibiotic at the concentration that reduced the titre of spores produced to less than $2 \times 10^3 \text{ ml}^{-1}$ (except for rifampin where it was 10^4 ml^{-1}). Because pppGpp accumulated in parallel to ppGpp but always had a lower

Fig. 3. Changes in the intracellular concentrations of ppGpp (a) and GTP (b) after cell transfer to Omv-medium containing certain antibiotics. Cells of strain 61886 were grown and transferred to Omv-medium containing different antibiotics and extracted as in Fig. 2. ●, Omv-medium alone; ○, plus 5 µg puromycin ml⁻¹; ■, plus 0.15 µg erythromycin ml⁻¹; □, plus 0.025 µg actinomycin ml⁻¹; ▲, plus 9 µg lincomycin ml⁻¹; △, plus 1.2 µg tetracycline ml⁻¹; ▼, plus 12 µg spectinomycin ml⁻¹; ▽, plus 0.37 µg thiostrepton ml⁻¹; ◇, plus 80 µg streptomycin ml⁻¹.

concentration, only the results for ppGpp are given in Figs 2 to 4. Fusidate (Fig. 2), lincomycin, and tetracycline (Fig. 3) prevented the (p)ppGpp accumulation almost completely. Chloramphenicol (Fig. 2b) enabled a transient accumulation of ppGpp to only 13 pmol per absorbance unit, which is about 20% of the value of the control not containing antibiotic. Therefore, these four compounds significantly inhibited the stringent response. Streptomycin (Fig. 3) reduced ppGpp accumulation to 40 pmol per absorbance unit, whereas the remaining compounds (actinomycin, erythromycin, puromycin, spectinomycin and thiostrepton) allowed accumulation of ppGpp to more than 50 pmol per absorbance unit; thus the inhibitory effect observed at low concentrations of these antibiotics did not interfere with the stringent response. At four times higher concentrations some of these compounds (erythromycin, puromycin, streptomycin and thiostrepton, but not spectinomycin) also reduced the ppGpp accumulation to less than 17 pmol per absorbance unit (data not shown), which indicates that they then also interfered (directly or indirectly) with the stringent response.

The intracellular concentration of GTP did not decrease if that of (p)ppGpp did not increase. When one of the three antibiotics that prevented (p)ppGpp accumulation was present, the concentration of GTP actually increased during amino acid deprivation (for fusidate, see Fig. 2; for lincomycin and tetracycline, see Fig. 3). However, when decoyinine was added along with fusidate, the concentration of GTP decreased essentially as much as in the absence of both the antibiotic and decoyinine (Fig. 2). Chloramphenicol addition reduced the decrease in the concentration of GTP to a level apparently not sufficient to induce significant sporulation (at higher concentrations, chloramphenicol produced an accumulation of GTP); decoyinine addition, which restored sporulation, caused a further GTP decrease (Fig. 2d). For the other inhibitors of protein synthesis, the effect of decoyinine on GTP synthesis was not measured because the compound restored sporulation only to a limited extent.

Fig. 4. Changes in the intracellular concentration of ppGpp (a) and GTP (b) after cell transfer to Omv-medium containing inhibitors of RNA polymerase. Cells of strain 61886 were grown, transferred to Omv-medium containing the antibiotics (plus or minus decoyinine), and extracted as in Fig. 1. Omv-medium: plus $0.03 \mu\text{g rifampin ml}^{-1}$ (●); plus $0.03 \mu\text{g rifampin ml}^{-1}$ plus $0.3 \text{ mM-decoyinine}$ (□); plus $0.5 \mu\text{g lipiarmycin ml}^{-1}$ (▲); plus $0.5 \mu\text{g lipiarmycin ml}^{-1}$ plus $0.3 \text{ mM-decoyinine}$ (■).

Addition of one of the RNA polymerase inhibitors, lipiarmycin or rifampin, produced a different response (Fig. 4). Although these compounds did not prevent (p)ppGpp accumulation, they caused a drastic GTP accumulation; the other three ribonucleoside triphosphates also accumulated (data not shown). Even decoyinine addition did not prevent the initial accumulation of GTP (Fig. 4b), but in its presence, the concentration of GTP eventually decreased to a value below $150 \text{ pmol per absorbance unit}$, the critical GTP level below which massive sporulation is initiated in this strain (61886) (Ochi *et al.*, 1981).

The results above showed that in the presence of some of the antibiotics, the concentration of GTP decreased to the critical value of about $150 \text{ pmol per absorbance unit}$ needed for massive sporulation initiation, only several hours after decoyinine addition. Therefore, one would expect that sporulation would be correspondingly delayed, as demonstrated in Fig. 5. The time delay between sporulation without antibiotic addition and sporulation in the presence of both antibiotic and decoyinine was about 2 h for fusidate, and more than 4 h for rifampin. These delays were longer than the time required to decrease GTP below the critical level, which indicates that other cellular reactions also had to adapt to the inhibitory effect of the antibiotics.

Use of antibiotic-resistant mutants

Most antibiotics reacting with ribosomes inhibit protein synthesis only if a certain ribosomal protein or RNA component has its normal structure. Mutants whose growth is resistant to the antibiotic are sometimes altered or deficient in that ribosomal component or in an enzyme normally used for its modification. We have used three such mutants resistant to erythromycin, lincomycin or thiostrepton, to determine whether the same cellular (ribosomal) structure needed for the antibiotic inhibition of growth was also needed for the inhibition of sporulation. In the antibiotic-sensitive strain 61886 decoyinine induced excellent sporulation; but if the medium also contained any one of the three antibiotics listed above, sporulation was abolished (Table 2). In contrast, each antibiotic-resistant mutant sporulated in the presence of the antibiotic to which it was resistant but could not sporulate in the presence of either of the other two antibiotics. We also examined sporulation in another stringent strain (61653) and a relaxed (*relA*) strain (61885); all three antibiotics prevented sporulation in these strains (Table 2). Thus a

Table 2. *Effect of antibiotics on the sporulation of antibiotic resistant strains*

The strains were grown at 37 °C in synthetic medium containing excess of required compounds (see Methods). For strain 61953, the medium was supplemented with 0.3% (w/v) vitamin-free casein hydrolysate. When the A_{600} was 0.5, 10 ml portions were transferred into flasks containing an amount of decoyinine that produced optimal sporulation (0.4 mM-decoyinine for 61885 and 62100, 2 mM for 61653, 61885 and 61953, and 4 mM for 62236) and various amounts of each antibiotic. The cultures were shaken for 14 h at 37 °C, whereupon the titre of heat-resistant spores was measured.

Antibiotic added	Antibiotic concn ($\mu\text{g ml}^{-1}$)	No. of heat-resistant spores from strain:					
		61886 (rel ⁺)	61653 (rel ⁺)	61885 (relA)	62100 (ery)	62236 (lin)	61953 (asp = relC)
None		2.0×10^1	1.0×10^5	2.2×10^2	8.0×10^3	2.5×10^4	1.0×10^1
Decoyinine		1.1×10^8	1.2×10^8	1.0×10^7	1.3×10^8	1.4×10^7	4.1×10^6
Erythromycin and decoyinine	0.25	1.2×10^4	2.2×10^2	1.0×10^1	1.3×10^8	1.1×10^4	1.0×10^1
	1	1.0×10^1	2.0×10^2	1.0×10^1	1.0×10^8	1.0×10^1	1.0×10^1
	4	3.0×10^1	5.0×10^1	1.0×10^1	1.0×10^8	1.0×10^1	1.0×10^1
Lincomycin and decoyinine	10	8.0×10^3	5.0×10^4	2.2×10^2	2.8×10^4	2.5×10^7	1.0×10^1
	40	2.0×10^2	7.5×10^3	9.0×10^1	5.8×10^3	4.7×10^7	6.0×10^1
	100	1.0×10^1	7.0×10^1	1.0×10^1	1.0×10^1	1.0×10^7	1.0×10^1
Thiostrepton and decoyinine	0.8	2.5×10^3	1.0×10^3	1.0×10^1	1.0×10^1	2.7×10^4	4.7×10^6
	3	6.0×10^1	1.3×10^2	2.0×10^1	2.5×10^2	1.0×10^1	3.5×10^6
	10	5.0×10^1	1.0×10^1	1.0×10^1	2.9×10^2	1.0×10^1	4.1×10^6

Fig. 5. Time course of sporulation after cell transfer to Omv-medium containing different antibiotics and decoyinine. Cells of strain 61886 were grown in Ile-medium to $A_{600} = 0.5$ and transferred to Omv-medium containing antibiotics and decoyinine. At the indicated times, 2 ml portions were removed and their heat-resistant spore titre was measured. ●, Omv-medium alone; ○, plus $0.14 \mu\text{g}$ fusidic acid ml^{-1} and 0.3 mM -decoyinine; △, plus $0.03 \mu\text{g}$ rifampin ml^{-1} and 0.3 mM -decoyinine.

mutation making cell-growth resistant to a particular antibiotic also made the sporulation process resistant to that antibiotic.

DISCUSSION

In *Escherichia coli*, amino acid starvation results in the synthesis of (p)ppGpp if uncharged tRNA has attached to the acceptor site for aminoacyl-tRNA (A-site on the 70S ribosomal complex) and if the necessary enzyme (stringent factor) as well as the substrates GTP and ATP are available (Cashel, 1975). We assume the same mechanisms work in *B. subtilis*. Therefore, when sporulation was induced by sudden partial deprivation of Ile, a fraction of the tRNA_{Ile} molecules must have been uncharged; as we have shown here and earlier (Ochi *et al.*, 1981), this caused a (transient) high accumulation of (p)ppGpp and a concomitant decrease in the concentration of GTP. The partially known effects of antibiotics on ribosomes and protein synthesis have been summarized by Nierhaus & Wittmann (1980) and can be used to explain our results. However, our antibiotic concentrations were much lower than those commonly used for *in vivo* and *in vitro* experiments. If different affinity constants or different biochemical effects have been reported, the effects at low antibiotic concentrations (high affinity constants) are relevant for this discussion. At higher antibiotic concentrations, at which protein synthesis (and growth) would be strongly inhibited, the stringent response would be avoided simply because charged Ile-tRNA could no longer be used and therefore would no longer be uncharged. This argument does not seem to apply to our conditions since most antibiotics were used at concentrations at which they inhibited growth by less than 50% and some of them reduced the accumulation of (p)ppGpp little or not at all (erythromycin, puromycin and spectinomycin). The latter three compounds inhibit ribosomal functions other than the binding of tRNA to the A-site (Nierhaus & Wittmann, 1980; Oleinick, 1975; Nathans, 1967; Wallace *et al.*, 1979). In contrast, compounds that reduced the accumulation of (p)ppGpp and the decrease of GTP may interfere (directly or indirectly) with the attachment of aminoacyl-tRNA (and therefore presumably also uncharged tRNA) to the A-site; such an interference has been observed for fusidate (Tanaka, 1973), tetracycline (Kaji & Ryoji, 1979) and a small effect for thiostrepton (Cundliffe, 1979) and streptomycin (Wallace *et al.*, 1979), and it has been suggested for chloramphenicol (Pongs, 1979) and lincomycin (Chang, 1979) from studies on the binding of small terminal fragments of tRNA.

The inhibition of sporulation caused by antibiotics that inhibited the stringent response could be counteracted, more or less efficiently, by the addition of decoyinine. This was most pronounced with chloramphenicol and fusidate and less with erythromycin, streptomycin, tetracycline and thiostrepton. The residual inhibition of sporulation, observed with some antibiotics in the presence of decoyinine, could be explained in two ways. (1) The normal structure of ribosomes is more important for differentiation (e.g. sporulation-specific protein synthesis) than for growth (general protein synthesis). Therefore, ribosomes to which these antibiotics have attached are not as efficient as normal ribosomes in allowing sporulation. Attachment of the antibiotic to a single ribosomal site can then explain both the interference with the stringent response and the residual interference with sporulation. (2) The antibiotic binds to two different sites only one of which interferes with the stringent response so that its effect can be overcome by decoyinine.

Because the lincomycin-resistant (*lin*) mutant could be induced by decoyinine to sporulate regardless of whether lincomycin was present or not, lincomycin apparently inhibited in the sensitive strains both growth and sporulation by binding to the same ribosomal component; this indicates a single site effect. Lincomycin has a specific effect also in *E. coli* and *Vibrio cholerae* where it stimulates an increase in the rate of toxin production (Levner *et al.*, 1980).

The single-site binding can also explain the effect of thiostrepton which binds to the 50S ribosomal subunit and inhibits (in *E. coli*) mainly the GTP hydrolysis associated with IF-2, EF-Tu and EF-G and the binding of aminoacyl-tRNA to the A-site (Cundliffe, 1979). Thiostrepton does not interfere only with the stringent response, because it also prevented sporulation even at a concentration of only $4 \mu\text{g ml}^{-1}$, at which it did not prevent the accumulation of (p)ppGpp. Thiostrepton-resistant mutants of *B. subtilis* are deficient in the ribosomal L11 protein and are relaxed (*relC*) (Smith *et al.*, 1980). Because decoyinine could induce sporulation of such a mutant in the presence of high thiostrepton concentrations, the inhibition of sporulation in the sensitive strain seems to result from thiostrepton binding to the same ribosomal site (involving the L11 protein) which is involved in the stringent response.

Erythromycin inhibited sporulation by binding to the 50S ribosomal subunit because a resistant mutant, known to be altered in ribosomal protein L17 (Tipper *et al.*, 1977), could be induced by decoyinine to sporulate well in the presence of erythromycin. This result as well as the earlier finding by Tipper *et al.* (1977) that erythromycin-resistant mutants are temperature sensitive for sporulation in nutrient medium shows that the normal structure of the L17 ribosomal protein is more important for sporulation than for growth (at least when sporulation is caused by the stringent response). This and other antibiotics, whose inhibition of sporulation could not be prevented by the addition of decoyinine, might preferentially inhibit the synthesis of proteins needed for sporulation; but their effect could also reflect the general finding that sporulation is much more sensitive to metabolic disturbances than is growth (Freese, 1981).

The addition of small amounts of rifampin or lipiarmycin, both inhibitors of RNA polymerase, to the resuspension medium had the unusual effect that GTP accumulated although the *de novo* GMP synthesis must have been inhibited by the simultaneously accumulating (p)ppGpp. This probably resulted from the inhibition of RNA synthesis because all nucleoside triphosphates accumulated. This accumulation apparently caused feed-back inhibition of further nucleotide synthesis because the nucleotide concentration eventually decreased again. Decoyinine inhibited GMP synthesis even more so that the cells eventually sporulated.

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