Effect of Gramicidin S on the Transcription System of the Producer
Bacillus brevis Nagano

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The effect of the peptide antibiotic gramicidin S, produced by Bacillus brevis Nagano, was tested on the transcription system of the producer by using in vivo, semi in vitro and in vitro systems for studies of RNA synthesis. The effects of other peptide antibiotics (linear gramicidin, tyrocidine and tyrothricin) were also tested for comparison. It was found that (a) RNA polymerase isolated from either gramicidin S-producing or non-producing strains had a similar structure and requirements and that (b) the presence of gramicidin S caused a very strong inhibition of the in vitro transcription system. We present evidence that this inhibition is most probably through formation of a complex between the antibiotic and the DNA. In vivo studies indicate that transcription during growth and sporulation is not affected by gramicidin S and the implication is made that gramicidin S inhibits transcription during germination and outgrowth.

INTRODUCTION

Bacilli produce peptide antibiotics at the onset of sporulation. There have been a number of suggestions as to whether or not these antibiotics play a regulatory role in the developmental cycle of their producers (Hodgson, 1970; Sadoff, 1972; Katz & Demain, 1977; Levinson et al., 1978). An attractive regulatory role of peptide antibiotics was proposed as a result of studies with B. brevis ATCC 8185 and the antibiotics tyrocidine and linear gramicidin produced by this strain. It was shown that tyrocidine could inhibit RNA synthesis in B. brevis ATCC 8185 in vivo and in vitro (Sarkar & Paulus, 1972a; Schaschneider et al., 1974; Ristow et al., 1975a). In the in vitro studies a complex was demonstrated between the antibiotic and the DNA (Schaschneider et al., 1974). On the other hand, linear gramicidin, which could also inhibit RNA synthesis in vitro (Ristow et al., 1975b; Paulus & Sarkar, 1976), apparently by interacting specifically with the DNA-dependent RNA polymerase (Sarkar et al., 1979), could weaken the DNA–tyrocidine complex, operating antagonistically to tyrocidine (Ristow, 1977; Chakraborty et al., 1978). Since both antibiotics are produced by the same strain in B. brevis they were suggested as possible regulators of gene expression during differentiation in their producer (Sarkar & Paulus, 1972a; Ristow et al., 1979).

Gramicidin S is a cyclic decapetide produced by B. brevis strains that do not produce tyrocidine or linear gramicidin. Although the mechanism of gramicidin S biosynthesis is well understood (Saito et al., 1970; Lipmann et al., 1971), the role of the antibiotic in the producer is still under investigation. Gramicidin S-negative mutants were isolated and identified (Iwaki et al., 1972; Shimura et al., 1974) and no significant relationship between antibiotic production and sporulation was found (Demain et al., 1976; Nandi & Seddon, 1978). In previous studies we have presented evidence that the antibiotic may have a role in spore outgrowth (Nandi & Seddon, 1978; Seddon & Nandi, 1978; Lazaridis et al., 1980), which was later verified by two

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more groups (Marahiel et al., 1979; Piret & Demain, 1982). In the present paper we report a detailed study of the effect of gramicidin S on the transcriptional apparatus of the producer organism, B. brevis Nagano, testing the possibility that it could act in a manner similar to tyrocidine or linear gramicidin on its producer organism, B. brevis ATCC 8185. On the basis of our findings from measurements in whole cells, toluene-treated cells (semi in vitro) and an in vitro system, we propose that gramicidin S could block transcription of the producer when present during germination and thus cause inhibition of outgrowth.

**METHODS**

**Bacterial strains, media, and culture conditions.** Bacillus brevis Nagano wild-type and gramicidin S-negative mutant E-1 were kindly supplied by Professor Y. Saito, Hyogo College of Medicine, Japan. Growth and sporulation took place in nutrient broth medium (Oxoid) at a concentration of 25 g l⁻¹ adjusted to pH 7.5. All other conditions were as described earlier by Nandi & Seddon (1978). Growth was monitored in a Pye Unicam SP 1800 spectrophotometer by measuring the optical density at 600 nm and sporulation was followed under a phase contrast microscope by counting the percentage of bacteria containing phase bright spores.

**Addition of antibiotic to cultures.** Gramicidin S, tyrocidine, tyrothricin, and linear gramicidin were added as ethanolic solutions (less than 0.5%, v/v) to a final concentration of 120 µg ml⁻¹. Equivalent volumes of ethanol were added to control incubations with no resultant effect on growth, sporulation, or the incorporation of radioactive precursors.

**Measurement of incorporation of radioactive precursors.** Cultures of E-1 or wild-type with or without antibiotic were incubated 5 min after addition of the antibiotic with either [5-¹⁴C]uracil [0.1 µCi ml⁻¹ (3.7 kBq ml⁻¹); 50–60 mCi mmol⁻¹] or [6-¹⁴]hypoxanthine (1 µCi ml⁻¹; 21 Ci mmol⁻¹) or 1L[U-¹⁴C]lysine (0.2 µCi ml⁻¹; 330 mCi mmol⁻¹). At set times, samples were transferred to an equal volume of 10% (w/v) ice-cold TCA, and left on ice for at least 30 min. The precipitates were collected on glass fibre filters (Whatman GF/C), washed three times with 5% (w/v) TCA and three times with ethanol, dried, and their radioactivity determined in a liquid scintillation counter.

**Tolueneized cell system.** Toluene treatment was based on the method described by Fisher et al. (1975) and Rothstein et al. (1976). Vegetative cells (30 ml) in mid-exponential phase (OD₆₀₀ = 1), or an equivalent number of cells at other stages of growth, were used for each assay. Cells were collected by centrifugation at room temperature at 12000 g for 3 min, washed with 2 vols ice-cold 0.05 M-Tris/HCl buffer, pH 7.9, by pipetting the cold buffer directly onto the cell pellet and shaking vigorously for 30 s. The washed cells were immediately collected by centrifugation at 4°C at 12000 g for 3 min, and resuspended in 1 ml 15% (v/v) toluene in ice-cold 0.05 M-Tris/HCl, pH 7.9 (6 × 10⁴ cells ml⁻¹). The cells were vortex mixed for 30 s and incubated on ice for 20 min before assay for [³²P]UTP incorporation into TCA-precipitable material. Reaction tubes contained in a total volume of 1.0 ml: 0.04 M-Tris/HCl pH 7.9; 0.3 mm (each) of GTP, UTP and CTP; 1.5 mM-ATP; 0.2 mM-dithiothreitol; 0.1 M-KCl, 0.01 M-MgCl₂; 1 mM-EDTA; and 2 µCi [5,6-¹⁴C]UTP ml⁻¹ (40–60 Ci mmol⁻¹). The assays were performed at 37 °C. Samples (100 µl) were removed at set times and added to 2 ml ice-cold 5% (w/v) TCA containing 0.01 M-sodium pyrophosphate, left on ice for 30 min, the precipitate collected on glass fibre filters (Whatman GF/C), washed with 5% (w/v) TCA and three times with ethanol, dried, and their radioactivity determined in a liquid scintillation counter. Gramicidin S, tyrocidine and linear gramicidin were added as ethanolic solutions (less than 0.5%, v/v) and actinomycin D and rifampicin as aqueous solutions.

**RNA polymerase purification.** All steps were carried out at 4°C and all buffers were prepared with deionized water. Vegetative cells (OD₆₀₀ = 1) were harvested by centrifugation at 12000 g for 3 min and washed with 4 vols ice-cold 0.05 M-Tris/HCl buffer, pH 7.9. The washed cells were immediately collected by centrifugation at 12000 g for 3 min and the packed cell volume was resuspended in 4 vols buffer A (10 mM-Tris/HCl pH 8; 10 mM-2-mercaptoethanol; 1 mM-EDTA; 15%, v/v, glycerol; 1 mM-phenylmethylsulphonylfluoride) and broken in an MSE 150 W ultrasonic disintegrator with 10 × 5 sonication pulses. The extract (fraction I) was centrifuged at 120000 g for 30 min and the supernatant solution (fraction II) was taken to 35% (w/v) ammonium sulphate saturation. After centrifugation at 120000 g for 30 min the supernatant was taken to 45% (w/v) saturation with ammonium sulphate, centrifuged at 90000 g for 30 min and the supernatant taken to 60% (w/v) saturation with ammonium sulphate. The enzyme was precipitated by centrifugation at 90000 g for 20 min (fraction III). The material which precipitated with 60% (w/v) ammonium sulphate was diluted in buffer A until the solution had a conductivity similar to that of buffer A + 0.025 M-KCl [10–1.5 ml (mg protein)⁻¹] and was chromatographed on a 1.6 × 10 cm column of DEAE-cellulose with a linear gradient from 0.025 to 0.5 M-KCl in buffer A running at 50 ml h⁻¹. DEAE-cellulose and agarose columns were treated as described by Burgess (1969) and Burgess & Travers (1971). Active fractions from DEAE-cellulose (fraction IV) were combined, precipitated with 70% (w/v) ammonium sulphate, and centrifuged at 10000 g for 30 min. The pellet was dissolved in buffer B (0.05 M-Tris/HCl pH 7.9; 0.1 mM-EDTA; 0.1 mM-dithiothreitol; and 15%, v/v, glycerol) and applied on a 2.6 × 85 cm agarose column (Bio-gel A-5 m, 200–400 mesh) which was eluted with an upward flow at 10 ml h⁻¹, with buffer B. Active
fractions from agarose were pooled (Fraction V) and concentrated by dialysis against buffer A + 70% (w/v) ammonium sulphate overnight at 4°C. The dialysed sample was centrifuged at 10000 g for 30 min and the pellet was again resuspended in 4 vols buffer and the dialysis and centrifugation steps repeated once more. The pellet obtained after the second centrifugation was dissolved in buffer A + 50% (v/v) glycerol at 5 mg ml⁻¹ and was stored at -20°C.

Typically 26 g of wild-type strain cells with an original specific activity of 0.8 units (mg protein)⁻¹ (units expressed as nmol UMP incorporated in 15 min under conditions described in the enzyme assay below) present in the 120000 g supernatant yielded a specific activity of 210 units (mg protein)⁻¹ for fraction V when herring sperm DNA was used as template. Somewhat lower [100 units (mg protein)⁻¹] and higher values [363 units (mg protein)⁻¹] were obtained with calf thymus DNA and poly dAT as template respectively. Similar observations were made for the enzyme purified from the gramicidin S-negative mutant E-1 using herring sperm DNA as template. The total activity present increased from around 600 units for the 120000 g supernatant to 1000 units for fraction V. Whether or not this reflects activation of the enzyme or removal of inhibitor substances is not known.

Enzyme assay. RNA polymerase was assayed at 37°C for 15 min in the presence of 30 mM-Tris/HCl, pH 7.9, 1 mM (each) ATP, GTP and CTP, 10 mM-MgCl₂, 0.1 mM [5,6-³²P]UTP, (10 μCi mmol⁻¹), 2 mM-MnCl₂, 10 mM-2-mercaptoethanol, 0.5 mg BSA ml⁻¹, 0.1 mg DNA ml⁻¹ and 0.4 mM-potassium phosphate, pH 7.5, in a final volume of 0.2 ml. When E. coli RNA polymerase was assayed 0.15 m-KCl was added to the assay mixture. The reaction was terminated by the addition of 2 ml ice-cold 5% (w/v) TCA containing 0.01 m-sodium pyrophosphate and left on ice for 30 min. The precipitate was collected on glass fibre filters (Whatman GF/C) previously soaked in 2% (w/v) TCA containing 0.01 m-sodium pyrophosphate and washed three times with the same mixture and three times with ethanol. The radioactivity was measured in a liquid scintillation counter. Antibiotics to be tested were added to parallel assays. Carrier solvents comprised less than 1% (v/v) of the total volume and when added alone had no effect.

Protein determination. Protein was measured by the procedure of Lowry, using crystalline BSA as standard. Prior to measurement, the proteins were precipitated with 10% (w/v) TCA as specified by Jendrisak & Burgess (1975). The protein concentration of active column fractions was determined from the absorbance at 280 nm and by assuming ε₂₈₀ = 6.6 (Richardson, 1966).

Polyacrylamide gel electrophoresis. Active fractions were analysed by electrophoresis on polyacrylamide gels containing 0.1 m-sodium phosphate pH 7.2, 0.1% (w/v) SDS, 5% (w/v) acrylamide, and 0.135% (w/v) N,N'-methylenebisacrylamide as described by Shapiro et al. (1967). The gels (6 x 0.5 cm) were run for 2.5-3 h, stained for 30 min at 50°C in a 0.2% (w/v) Coomassie brilliant blue solution in methanol/acetic acid/water (5:1:5, by vol.), rinsed in 7.5% (v/v) acetic acid plus 5% (v/v) methanol and destained in this solution at 50°C.

Samples were dried down in a vacuum desiccator or under an air stream and in cases where they contained glycerol they were first dialysed against distilled water. 10 μl 0.05% (w/v) bromophenol blue in 50% (v/v) glycerol and 45 μl sample buffer (1%, w/v, SDS; 1%, w/v, 2-mercaptoethanol; 0.01 m-sodium phosphate buffer pH 7.2) were added and the sample was boiled for 2 min. Electrode buffer was gel buffer diluted 1:1 with distilled water. The gels were scanned with a Unicam scanning densitometer and the relative amounts of the stained bands were estimated from the densitometer tracing. Molecular weight markers were run in parallel gels.

Isolation of DNA. B. brevis Nagano DNA was isolated by the method of Marmur (1961). The same method was used for [³²P]DNA isolation. In this case cells were grown in the presence of 4 x 10⁻³ μCi [³²P]thymidine ml⁻¹ (28 Ci mmol⁻¹) for several generations before DNA extraction. The specific activity of the extracted [³²P]DNA was found to be 10⁴ c.p.m. (mg DNA)⁻¹.

Materials. Gramicidin S and linear gramicidin were purchased from Sigma; tyrocidine and tyrothricin from Nutritional Biochemical Corporation, Cleveland, Ohio, USA; ATP from Boehringer Mannheim; CTP, GTP and UTP from Sigma. DEAE-cellulose (preswollen, microgranular, DE-52) was obtained from Whatman, and agarose (A-5m, 200–400 mesh) from Bio-Gel. Bovine albumin, ovalubamin, lysozyme and immunoglobulin G were from Sigma; β-galactosidase, E. coli RNA polymerase and carboxypeptidase were from Boehringer Mannheim, and trypsin was from Hopkin and Williams, Chadwell Heath, Essex, UK. Calf thymus DNA and E. coli DNA were from Sigma; herring sperm DNA and poly dAT were from Boehringer Mannheim. Radioactively labelled compounds were from Amersham except for [¹⁴C]gramicidin S which was a gift from Dr I. Lazaridis, Department of Biology, University of Ioannina, Greece.

RESULTS

Studies in whole cells

Both B. brevis Nagano wild-type and the gramicidin S-negative mutant E-1 showed similar trends of growth and sporulation (50–60% sporulation at T₁₀, Fig. 1) in nutrient broth medium. These trends were not altered by addition to the culture at the end of the growth phase...
Fig. 1. [3H]UTP incorporation by toluenized *B. brevis* Nagano cells during growth and sporulation. ●, Growth; ○, sporulation; □, [3H]UTP incorporation by E-1; ■, [3H]UTP incorporation by wild-type.

(OD<sub>600</sub> = 1.5) of either gramicidin S or any of the antibiotics produced by other strains of *B. brevis*, that is linear gramicidin, tyrocidine, or tyrothricin at concentrations corresponding to those produced during sporulation (120 µg ml<sup>-1</sup>). Antibiotic addition to cells in the growth phase (OD<sub>600</sub> = 0.1, 1.0) or to cells at the beginning of sporulation (OD<sub>600</sub> = 1.9-2.2) did not affect growth or sporulation either. This was true for both E-1 and wild-type cultures.

In agreement with these results, the incorporation of radioactive uracil, thymidine, or lysine, measured at mid-exponential phase was unaffected by addition of antibiotics at 120 µg ml<sup>-1</sup>. Challenging the cultures with antibiotics at different times in growth and early sporulation (OD<sub>600</sub> = 0.1, 0.2, 0.4, 0.6, 0.8, 1.3, 1.5, 2.2, T<sub>2</sub>, T<sub>6</sub>, T<sub>8</sub>) also had no effect on the incorporation of macromolecular precursors. Moreover, prolonging the time of incubation of the culture with the antibiotics for up to 2.5 h before labelling, made no difference in the incorporation of [14C]uracil, [3H]thymidine, or [14C]L-lysine by E-1 or wild-type cultures, into acid-precipitable material.

**Studies in toluene-treated cells**

Vegetative cells were tested for the effect of gramicidin S, tyrocidine or linear gramicidin on [3H]UTP incorporation after toluenization. It was found that the three *B. brevis* antibiotics left the incorporation of [3H]UTP unaffected when added at 120 µg ml<sup>-1</sup> to mid-exponential phase of either E-1 or wild-type cultures, although the incorporation was both actinomycin D- and rifampicin-sensitive (Fig. 2). [3H]UTP incorporation tested at other stages of vegetative growth and sporulation of E-1 and wild-type bacteria (OD<sub>600</sub> = 0.1, 0.2, 0.4, 0.6, 0.8, 1.3, 1.5, 2.2, T<sub>2</sub>, T<sub>6</sub>, T<sub>8</sub>) was also unaffected by gramicidin S, tyrocidine or tyrothricin.

A marked decrease in transcriptional activity occurred as the cells approached stationary phase (Fig. 1). This decrease did not lead to a complete cessation of RNA synthesis since addition of rifampicin (0.1 mg ml<sup>-1</sup>) to either wild-type or E-1 cultures at T<sub>6</sub> of sporulation (when only 5% phase bright spores were present) prevented the normal increase to 60–70% sporulation observed in these cultures at T<sub>10</sub> and after in the absence of rifampicin. Sporulation in both E-1 and wild-type cultures did not become rifampicin-insensitive until T<sub>7</sub> of sporulation. This agrees with the literature where RNA synthesis during sporogenesis is well documented (Sonenshein & Campbell, 1978).

**Composition and properties of RNA polymerase**

For studies of *in vitro* transcription, RNA polymerase was purified from *B. brevis* Nagano as described in Methods. Electrophoresis of the purified preparation of RNA polymerase (Fraction V) in the presence of SDS revealed three major and two minor components for both E-1 and wild-type preparations (Fig. 3a). The molecular weights of these components were
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Fig. 2. Effect of antibiotic addition of [$^3$H]UTP incorporation by toluenized *B. brevis* Nagano cells. Cultures of either E-1 or wild-type were allowed to reach OD$_{600}$ = 1 and were then treated with toluene. The assay procedure was carried out as described in Methods. ○, Control, no additions; ■, 1 µg actinomycin D ml$^{-1}$; □, 1 µg rifampicin ml$^{-1}$; ▲, 120 µg gramicidin S ml$^{-1}$; △, 120 µg linear gramicidin or tyrocidine ml$^{-1}$.

Fig. 3. (a) Densitometer tracing of *B. brevis* Nagano RNA polymerase and estimation of the molecular weights of the enzyme subunits. The recorder response for 56µg enzyme in a SDS gel. The arrow indicates the direction of migration. (b) Proteins of known molecular weight as well as purified RNA polymerase were dissociated and subjected to electrophoresis through 5% (w/v) acrylamide gels as described in Methods and their mobilities relative to the tracking dye determined. The marker proteins were: *E. coli* RNA polymerase (10 or 1 µg), mol. wts of subunits $\beta = 160000, \sigma = 95000, \alpha = 39000$; $\beta$-galactosidase (50, 10 or 5 µg), mol. wt 130000; immunoglobulin G, heavy chain (16 or 3 µg), mol. wt 55000; bovine albumin (16 or 3 µg), mol. wt 68000; ovalbumin (25 or 5 µg), mol. wt 45000; carboxypeptidase (50 or 10 µg), mol. wt 34600; trypsin (50 or 5 µg), mol. wt 23300; lysozyme (50 or 10 µg), mol. wt 14300. The mobilities of the subunits of *B. brevis* RNA polymerase are indicated by arrows.

determined by comparing their electrophoretic mobilities with those of marker proteins of known molecular weight (Fig. 3b), and the molar ratios were calculated from the densitometer tracings. The major components had approximate molecular weights of 150000 ± 10000; 65000 ± 5000, and 38000 ± 1000, and occurred in molar ratios of about 2:6:1:3:3 ± 0-4, thus corresponding well to the $\beta + \beta'$, $\sigma$, and $\alpha$ subunits of RNA polymerase from *B. brevis* ATCC 8185 (Sarkar & Paulus, 1972b) and *B. subtilis* (Avila et al., 1971). Subunit $\alpha$ was found in higher ratios than subunits $\beta + \beta'$ in a number of preparations, and this was attributed to impurities running very close to the $\alpha$ band of the gels (Fig. 3a). From the densitometer tracings, the enzyme was calculated to be about 70–75% pure. The minor components of molecular weights 120000 and 95000 which were found in this preparation of RNA polymerase have also been reported in purification procedures of *B. brevis* ATCC 8185 (Sarkar & Paulus, 1972b, Paulus & Sarkar, 1976) and *B. subtilis* (Losick et al., 1970).
Table 1. **Requirements of RNA polymerase activity**

56 μg enzyme was used per assay. All values are the means of duplicate assays.

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>DNA template . . .</th>
<th>Calf thymus</th>
<th>Herring sperm</th>
<th>Poly dAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Template</td>
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<td>27100</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>12800</td>
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</tr>
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<table>
<thead>
<tr>
<th></th>
<th>Calf thymus</th>
<th>Herring sperm</th>
<th>Poly dAT</th>
</tr>
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<tbody>
<tr>
<td>B. brevis wild-type</td>
<td>9100</td>
<td>20400</td>
<td>26500</td>
</tr>
<tr>
<td>B. brevis E-1</td>
<td>170</td>
<td>150</td>
<td>180</td>
</tr>
</tbody>
</table>

ND, Not determined.

* The assay conditions for the complete system were as follows: 30 mM-Tris/HCl (pH 7.9), 1 mM (each) ATP, GTP and CTP, 10 mM-MgCl₂, 0-1 mM-[5,6-³H]U TP (10 μCi mmol⁻¹), 2 mM-MnCl₂, 10 mM-2-mercaptoethanol, 0.5 mg BSA ml⁻¹, 0.1 mg DNA ml⁻¹ and 0.4 mM-potassium phosphate (pH 7.5) in a final volume of 0.2 ml. The assay was carried out at 37 °C for 15 min.

During purification, RNA polymerase activity was found to be completely dependent on added template after the ammonium sulphate precipitation (Fraction III), and linearly dependent on the amount of enzyme protein added up to 300 μg ml⁻¹ with both calf thymus DNA, while it decreased to about 30% at pH 10. KCl at concentrations above 0.05 M and up to activity are shown in Table 1. Omission of any of the individual triphosphates other than CTP gave background counts. Assays without CTP retained about 20% of the activity, compared with the complete system, when either calf thymus DNA or herring sperm DNA was used as template (Table 1). A variation in the requirements for one of the triphosphates compared with the others has been reported for RNA polymerase purified from *B. subtilis* (Avila et al., 1971; Losick & Sonenshein, 1969) and *B. megaterium* (Chambon et al., 1968a).

Maximum activity of the enzyme was found at pH 7.5-8 for both herring sperm or calf thymus DNA, while it decreased to about 30% at pH 10. KCl at concentrations above 0.05 M and up to 0.2 M gave partial inhibition of RNA polymerase activity. KCl at 0.05 M or lower concentration was not inhibitory under our conditions, contrary to reports for *B. subtilis* RNA polymerase (Avila et al., 1971). The nature of the enzyme and/or the template have been reported to alter the effect of KCl on the activity (Linn et al., 1975). The effect of Mg²⁺ or Mn²⁺ ions was such that the maximum rate of synthesis was obtained at concentrations of 10 mM-MgCl₂ and 2 mM-MnCl₂. Incorporation of [³H]UMP was linear for a 20 min period. Heat-treatment of the enzyme for a 5 min period before the assay resulted in a loss of 50% activity when a temperature of 50 °C was used and almost complete loss of activity occurred at 60 °C.

*B. brevis* RNA polymerase transcribed herring sperm DNA more efficiently than calf thymus DNA throughout the purification procedure. The enzyme showed similar activity in transcribing *B. brevis* DNA, but lower levels of activity were obtained with *E. coli* DNA (Table 2). Sarkar et al. (1977) also reported that RNA polymerase purified from *B. brevis* ATCC 8185 showed similar efficiencies when transcribing salmon sperm DNA and *B. brevis* DNA.

**Inhibition of RNA polymerase in vitro by *B. brevis* peptide antibiotics**

Irrespective of the template used, addition of 20 μg gramicidin S to the transcriptional assay strongly inhibited enzyme activity (Table 2). Gramicidin S was a very potent inhibitor of enzyme activity in preparations of either E-1 or wild-type or commercial *E. coli* RNA polymerase, reducing the activity to 10-20% of the corresponding control (Table 3). Tyrocidine (20 μg per assay) was also a potent inhibitor of both *B. brevis* and *E. coli* RNA polymerase.
Gramicidin S and transcription

Table 2. Activity of RNA polymerase with several templates in the presence and absence of gramicidin S

The assays were performed as described in Methods. The values are the means of duplicate assays; 14 µg enzyme was used per assay.

<table>
<thead>
<tr>
<th>DNA template</th>
<th>Activity [acid-insoluble [3H]UTP (c.p.m.)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− Gramicidin S (20 µg) + Gramicidin S</td>
</tr>
<tr>
<td>Herring sperm</td>
<td>4200 720</td>
</tr>
<tr>
<td>E. coli</td>
<td>3500 510</td>
</tr>
<tr>
<td>B. brevis</td>
<td>4700 450</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>2900 280</td>
</tr>
<tr>
<td>Poly dAT</td>
<td>7500 840</td>
</tr>
</tbody>
</table>

Table 3. Effect of antibiotics on RNA polymerase activity

The assays were performed as described in Methods and values given are the means of duplicate assays. B. brevis antibiotics were added at 100 µg ml⁻¹ and actinomycin D and rifampicin were added at 5 µg ml⁻¹. The amount of enzyme protein in each assay was 9 µg for B. brevis wild-type RNA polymerase, 1 µg for B. brevis E-1 RNA polymerase and 1.6 µg for commercial E. coli RNA polymerase.

<table>
<thead>
<tr>
<th>DNA template</th>
<th>Source of RNA polymerase</th>
<th>Gramicidin S</th>
<th>Linear gramicidin</th>
<th>Tyrocidine</th>
<th>Tyrothricin</th>
<th>Rifampicin</th>
<th>Actinomycin D</th>
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</thead>
<tbody>
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<td>Calf thymus</td>
<td>B. brevis wild-type</td>
<td>9</td>
<td>88</td>
<td>10</td>
<td>90</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>B. brevis mutant E-1</td>
<td>22</td>
<td>100</td>
<td>27</td>
<td>72</td>
<td>3</td>
<td>2</td>
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<tr>
<td></td>
<td>E. coli (commercial)</td>
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<td>98</td>
<td>7</td>
<td>28</td>
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<tr>
<td></td>
<td>B. brevis mutant E-1</td>
<td>14</td>
<td>103</td>
<td>41</td>
<td>66</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>E. coli (commercial)</td>
<td>6</td>
<td>93</td>
<td>6</td>
<td>50</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Poly dAT</td>
<td>B. brevis wild-type</td>
<td>3</td>
<td>90</td>
<td>3</td>
<td>39</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>B. brevis mutant E-1</td>
<td>14</td>
<td>95</td>
<td>15</td>
<td>34</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>E. coli (commercial)</td>
<td>2</td>
<td>44</td>
<td>1</td>
<td>22</td>
<td>2</td>
<td>–</td>
</tr>
</tbody>
</table>

*Control values, in the absence of antibiotic, are 100%

activity (Table 3), while the addition of 20 µg linear gramicidin per assay was found to inhibit only E. coli RNA polymerase transcribing on poly dAT. Tyrothricin, the mixture of linear gramicidin and tyrocidine, added at 20 µg per assay, caused little or no inhibition of B. brevis polymerase but this mixture was a strong inhibitor of E. coli polymerase with all templates (Table 3). Under the same conditions transcriptional activity was completely template-dependent and sensitive to actinomycin D and rifampicin.

The results presented in Table 3 were not altered when less-purified preparations of B. brevis RNA polymerase were assayed in the presence of antibiotics. Table 4 is a composite of results obtained when B. brevis antibiotics were added to transcriptional assays of B. brevis RNA polymerase at various stages of purification, and the results with E. coli RNA polymerase are included for comparison. Thepercentage inhibition of the fractions throughout the purification procedure was comparable for all three templates and was not related to the amount of enzyme used in the assays. Only tyrothricin showed higher inhibition of E. coli RNA polymerase than of B. brevis RNA polymerase. Linear gramicidin showed inhibition only when the synthetic template poly dAT was used.

In general, gramicidin S was found to be the most potent inhibitor irrespective of template or source of enzyme tested (87–95% inhibition) and tyrocidine also inhibited the enzyme activity quite strongly (55–96% inhibition). The greatest variation in the results was obtained with addition of tyrothricin (0–65% inhibition) with the greatest inhibition found when poly dAT
Table 4. Relationship of the template and/or the stage of purification of RNA polymerase to the inhibition of transcription by B. brevis peptide antibiotics

The assays were performed as described in Methods. The values (±SD) are the average of eight assays for B. brevis RNA polymerase, and four assays for E. coli RNA polymerase. Inhibition was measured as percentage acid-insoluble radioactivity incorporated with respect to the corresponding control.

<table>
<thead>
<tr>
<th>Source of RNA polymerase</th>
<th>Template DNA</th>
<th>Gramicidin S</th>
<th>Linear gramicidin</th>
<th>Tyrocidine</th>
<th>Tyrothricin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulphate fraction (50–70 µg)</td>
<td>Calf thymus</td>
<td>91 ± 5</td>
<td>-8 ± 11</td>
<td>79 ± 5</td>
<td>-9 ± 12</td>
</tr>
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<td></td>
<td>Herring sperm</td>
<td>89 ± 9</td>
<td>-8 ± 9</td>
<td>90 ± 8</td>
<td>20 ± 8</td>
</tr>
<tr>
<td></td>
<td>Poly dAT</td>
<td>90 ± 10</td>
<td>59 ± 12</td>
<td>93 ± 11</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>DEAE-cellulose agarose fraction (5–20 µg)</td>
<td>Calf thymus</td>
<td>94 ± 8</td>
<td>8 ± 8</td>
<td>89 ± 5</td>
<td>11 ± 6</td>
</tr>
<tr>
<td></td>
<td>Herring sperm</td>
<td>95 ± 7</td>
<td>-2 ± 6</td>
<td>96 ± 4</td>
<td>20 ± 10</td>
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<td></td>
<td>Poly dAT</td>
<td>88 ± 11</td>
<td>50 ± 5</td>
<td>95 ± 5</td>
<td>62 ± 11</td>
</tr>
<tr>
<td>Agarose fraction (1–9 µg)</td>
<td>Calf thymus</td>
<td>87 ± 6</td>
<td>7 ± 4</td>
<td>55 ± 10</td>
<td>29 ± 9</td>
</tr>
<tr>
<td></td>
<td>Herring sperm</td>
<td>87 ± 12</td>
<td>-11 ± 10</td>
<td>70 ± 7</td>
<td>5 ± 10</td>
</tr>
<tr>
<td></td>
<td>Poly dAT</td>
<td>92 ± 11</td>
<td>40 ± 9</td>
<td>92 ± 8</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>Commercial E. coli (1–6 µg)</td>
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<td>99 ± 2</td>
<td>0 ± 9</td>
<td>99 ± 1</td>
<td>75 ± 6</td>
</tr>
<tr>
<td></td>
<td>Herring sperm</td>
<td>92 ± 10</td>
<td>4 ± 12</td>
<td>92 ± 9</td>
<td>52 ± 7</td>
</tr>
<tr>
<td></td>
<td>Poly dAT</td>
<td>98 ± 4</td>
<td>58 ± 8</td>
<td>98 ± 3</td>
<td>77 ± 7</td>
</tr>
</tbody>
</table>

* Negative values refer to stimulation of incorporation with respect to the corresponding control.

was used as template for transcription. Linear gramicidin was least inhibitory (0–7% inhibition) with natural templates and was found partially to inhibit transcription on poly dAT template. These results were also true for the commercial preparation of E. coli RNA polymerase with perhaps the exception of tyrothricin, which gave higher inhibition with this enzyme.

Inhibition of transcription by gramicidin S and relief of inhibition by DNA

Inhibition of 9 µg purified wild-type B. brevis Nagano RNA polymerase transcribing about 6 µg B. brevis Nagano DNA by increasing amounts of gramicidin S showed that 50% inhibition can be achieved by 1 µg gramicidin S, indicating that the antibiotic is a strong inhibitor of transcription (Fig. 4a). For 50% inhibition of 1·6 µg E. coli RNA polymerase transcribing 20 µg herring sperm DNA 10 µg gramicidin S was needed, while 1 µg antibiotic gave 40% inhibition (Fig. 4b). However, addition of gramicidin S to the transcriptional assays after transcription had been allowed to proceed for some time left the incorporation unaffected, suggesting that the antibiotic could not prevent continuation of transcription once it had started.

Inhibition of RNA polymerase activity of B. brevis or E. coli by gramicidin S could be relieved proportionally by increasing amounts of DNA (Fig. 4c,d). On the other hand increasing the amounts of enzyme per assay did not reverse the inhibition by gramicidin S (Fig. 4c,d).

Complex formation between peptide antibiotics and B. brevis Nagano DNA

[3H]DNA isolated and purified from B. brevis Nagano was incubated with increasing concentrations of the four B. brevis antibiotics (Fig. 5). The degree to which the antibiotics were found to retain [3H]DNA on the filter was in good agreement with the results obtained from the transcription experiments (Table 4). Gramicidin S appears to have a greater ability to form a complex with B. brevis DNA than the other antibiotics tested. Use of [14C]gramicidin S showed that this affinity of gramicidin S was not due simply to the binding of the antibiotic on the filter. On the other hand, tyrocidine and linear gramicidin, which gave lower percentages of DNA retention than gramicidin S and no retention, respectively (Fig. 5), have been reported to stick to nitrocellulose filters (Ristow et al., 1975b).

Using the same technique we showed that no complex was formed between [14C]gramicidin S (6 or 12 µg) and RNA polymerase from either E. coli (1·6 µg) or B. brevis (9 µg), at least at the
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Fig. 4. Inhibition of RNA polymerase activity by gramicidin S and effects of DNA and RNA polymerase on transcription systems inhibited by gramicidin S. The assays were performed under the conditions specified in Methods. The values are the average of duplicate assays. Inhibition of (a) 9 µg RNA polymerase purified from *B. brevis* transcribing 6.2 µg *B. brevis* DNA, by increasing amounts of gramicidin S; (b) 1.6 µg commercial *E. coli* RNA polymerase transcribing 20 µg herring sperm DNA, by increasing amounts of gramicidin S. Effect of increasing amounts of (c) *B. brevis* DNA or RNA polymerase; (d) commercial *E. coli* DNA or RNA polymerase on inhibition by 20 µg gramicidin S.

Fig. 5. Complex formation between *B. brevis* DNA and antibiotics assayed by the filter binding technique of Riggs *et al.* (1970). [3H]DNA was isolated from *B. brevis* cultures as described in Methods. In total volumes of 1 ml, 100 µg [3H]DNA was incubated with increasing amounts of antibiotics for 10 min at room temperature in a buffer containing 0.01 M-Tris/HCl, pH 7.8, 0.01 M-magnesium acetate, 0.01 M-KCl, 0.1 mM-EDTA, 5% (v/v) dimethylsulphoxide and 50 µg BSA ml⁻¹. The mixture was then filtered slowly under a low vacuum through a nitrocellulose filter (Millipore) (HAMK 02412), previously soaked in the same buffer without BSA for 30 min, and was washed with 2 ml of the same buffer without albumin. The filter was dried and counted in a liquid scintillation counter. Under these conditions 10% of the DNA was retained on the filter in the absence of antibiotic.

concentrations tested. In contrast, the presence of either *E. coli* or *B. brevis* RNA polymerase caused 95% of the tritiated *B. brevis* DNA to be retained on the filters as would be expected. From these observations, a dual target for the antibiotic (DNA and RNA polymerase) seems unlikely.
DISCUSSION

The results from our studies on macromolecular synthesis with gramicidin S, linear gramicidin, tyrocidine or tyrothricin in whole cells and the semi in vitro system are in good agreement with other findings from our group that gramicidin S has no obvious effect on growth or sporulation of B. brevis Nagano wild-type and gramicidin S-negative mutants (Nandi & Seddon, 1978; Lazaridis et al., 1980; Nandi et al., 1981). However, the in vitro experiments demonstrated that gramicidin S inhibits the transcriptional system very strongly. This inhibition is most probably achieved through complex formation with the DNA template rather than through an interaction between the antibiotic and RNA polymerase, as was proposed for linear gramicidin (Fisher & Blumenthal, 1982), since the results here demonstrate an interaction between gramicidin S and DNA and not between gramicidin S and RNA polymerase. Complex formation between tyrocidine and B. brevis DNA is similar in our studies to that reported elsewhere (Ristow et al., 1975b), whereas gramicidin S showed a higher affinity than tyrocidine for DNA under the same conditions. This finding is in agreement with the mechanism of action we propose above. We also consider important the finding that the antibiotics are found to bind B. brevis Nagano DNA with an efficiency analogous to their ability to inhibit transcription in vitro (gramicidin S > tyrocidine > tyrothricin > linear gramicidin). From the information available these results could argue for a stronger affinity of gramicidin S for B. brevis Nagano template, since it has been reported that 10 μg gramicidin S can inhibit transcription in B. brevis ATCC 8185 (a non-producer of gramicidin S) only to about 23%, whereas tyrothricin, tyrocidine, or linear gramicidin (produced by the B. brevis ATCC 8185 strain) tested at the same concentration caused 96%, 100% and 90% inhibition of transcription respectively (Sarkar & Paulus, 1972a).

We have reported previously that gramicidin S, synthesized early in sporulation, although incorporated into the spore, does not seem to affect sporulation, or spore characteristics, but is found to exert a specific inhibition of outgrowth, possibly by inhibiting transcription during germination (Nandi & Seddon, 1978; Lazaridis et al., 1980; Nandi et al., 1985). The data presented here are consistent with the hypothesis that gramicidin S functions as an endogenous inhibitor of B. brevis Nagano outgrowth by complexing with the DNA during germination, thus preventing transcription necessary for outgrowth. There is evidence that the DNA undergoes conformational changes during sporulation and that it becomes associated with low molecular weight compounds which are thought to affect the template specificity of RNA polymerase (Orrego et al., 1973). These changes and associations resulted in the DNA being in a spore-specific state (Halvorson et al., 1966; Vanek & Mikulik, 1978) which needs to be reversed at germination to allow transcription (Stafford & Donellan, 1968; Matsuda & Kameyama, 1980). It has been reported that in Bacillus megaterium spores and vegetative cells, all the DNA is found associated with membranes (Chambon et al., 1968b). Olsen et al. (1974) state that in E. coli ‘DNA–envelope complex fragments’ are found to contain biochemical markers of membranes, and isolation of intact bacterial chromosomes by Portalier & Worcel (1976) revealed associations with membrane fragments. So, gramicidin S, if associated with the membranes of the spore in B. brevis Nagano (Nandi et al., 1981), could also be associated with DNA and thereby restrict the availability of the DNA for transcription. The idea of a tight packing of DNA in the spore, due to association with the peptide antibiotic, is supported by recently reported data from our own group and by others (Nandi et al., 1985; Hansen et al., 1982).

The lack of an effect of gramicidin S on vegetative and sporulating cells, when it is added to the culture, is taken as evidence that the antibiotic does not act during growth or sporulation whereas its inhibition of RNA synthesis and outgrowth when added at the germination/outgrowth stage indicates a specific role in the regulation of outgrowth (Seddon & Nandi, 1978; Nandi et al., 1985). It is unlikely that cells are impermeable to gramicidin S during growth and sporulation, since exogenously added gramicidin S, at these stages, is incorporated into the developing spore which itself is formed as a sporangial inclusion (Lazaridis et al., 1980). It could be that the DNA and the antibiotic are prevented from interacting during the growth phase, like edeine in the producer B. brevis Vm4 (Kurylo-Borowska & Szer, 1976), or that the DNA is in a conformational state that is not vulnerable to gramicidin S. Tyrocidine was reported to be
influenced in its capacity to bind DNA by the actual conformation of the DNA (Ristow et al., 1975c; Chakraborty et al., 1978). On the other hand, since gramicidin S is incorporated into the spore (Lazaridis et al., 1980) it may well form DNA-gramicidin S complexes with the spore DNA, whilst leaving the spore mother cell DNA free for sporulation-specific transcription. The state of the spore DNA and its availability for transcription would then be altered by complex formation with gramicidin S. DNA-gramicidin S complexes would prevent transcription and maintain the spore DNA in an inactive form, whereas dissociation of the DNA-gramicidin S complex, under conditions of germination suitable for outgrowth, would allow transcription to occur. [The possibility that this could be the result of a primary effect on active transport in vivo, as we had suggested earlier (Frangou-Lazaridis et al., 1980) either directly, as has been recently proposed (Danders et al., 1982) or indirectly, through an inhibition of respiration (Nandi et al., 1981) cannot be ruled out at this time, although it does appear from the results presented here that a direct interaction between gramicidin S and B. brevis DNA is more likely.]

If gramicidin S acts on transcription in the wild-type strain to inhibit outgrowth, then how does the producer evade its own antibiotic, as indeed it must do in order to survive? We have previously shown that at low spore population densities gramicidin S is rapidly lost from the spore during the early stages of germination and it is under these conditions that outgrowth will then proceed. It is only at high spore population densities that gramicidin S is not lost from the spore and outgrowth is inhibited (Lazaridis et al., 1980). Under such conditions of overcrowding, the germinating spores do indeed appear to commit suicide. However, not all the spores germinate at once; some remain dormant, and this may be a survival mechanism in that the remaining dormant spores can germinate later when environmental conditions are more favourable for subsequent growth. Just how the organism monitors the environment and by what control mechanism(s) gramicidin S is or is not lost from the spore is now under investigation.

REFERENCES


tural changes of superhelical DNA. European Journal of Biochemistry 126, 279–284.
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