EFFECTS OF THE PEPTIDE ANTIBIOTICS TYROCIDINE AND THE LINEAR GRAMICIDIN ON RNA SYNTHESIS AND SPORULATION OF BACILLUS BREVIS

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SUMMARY

Effects of the two peptide antibiotics tyrocidine and the linear gramicidin on exponential growing cultures of *Bacillus brevis* (ATCC 8185) were studied. Both antibiotics are produced by this bacterial strain. Our results demonstrate that the addition of tyrocidine leads to inhibition of RNA synthesis followed by a cessation of growth. In contrast, gramicidin does not affect net RNA synthesis and the cells continue to grow. However, sporulation is inhibited by gramicidin. The addition of a mixture of tyrocidine and gramicidin prevents partially the inhibitory effect of tyrocidine on RNA synthesis. The results presented are essentially in agreement with *in vitro* results described previously (8,9).

The natural function of the peptide antibiotics synthesized by sporulating bacteria is unknown, but a correlation between synthesis of the peptide antibiotics and sporulation has been proposed (1,2), and various experimental results suggest a regulatory role for these peptides (3-7).

We recently reported that the peptide antibiotic tyrocidine, isolated from the sporulating bacterial strain *Bacillus brevis* (ATCC 8185), interacts with DNA *in vitro* and inhibits RNA synthesis in a transcription assay, apparently by affecting the initiation process (8). Tyrocidine binds probably externally to the nucleic acid as concluded from melting experiments and spectrophotometric measurements (Schazschneider, Ristow, unpublished). When the linear peptide antibiotic gramicidin, which is produced by the same bacterial strain, was added to a tyrocidine-inhibited transcription system, RNA synthesis started again (9). Thus gramicidin abolishes the action of tyrocidine *in vitro*. The molecular mechanism of this reactivation is unknown, but might possibly reflect a regulative role of the two antibiotics in the cell (9).

Therefore we investigated the effects of tyrocidine and gramicidin on RNA synthesis and sporulation of growing cells of *Bacillus brevis*. We present

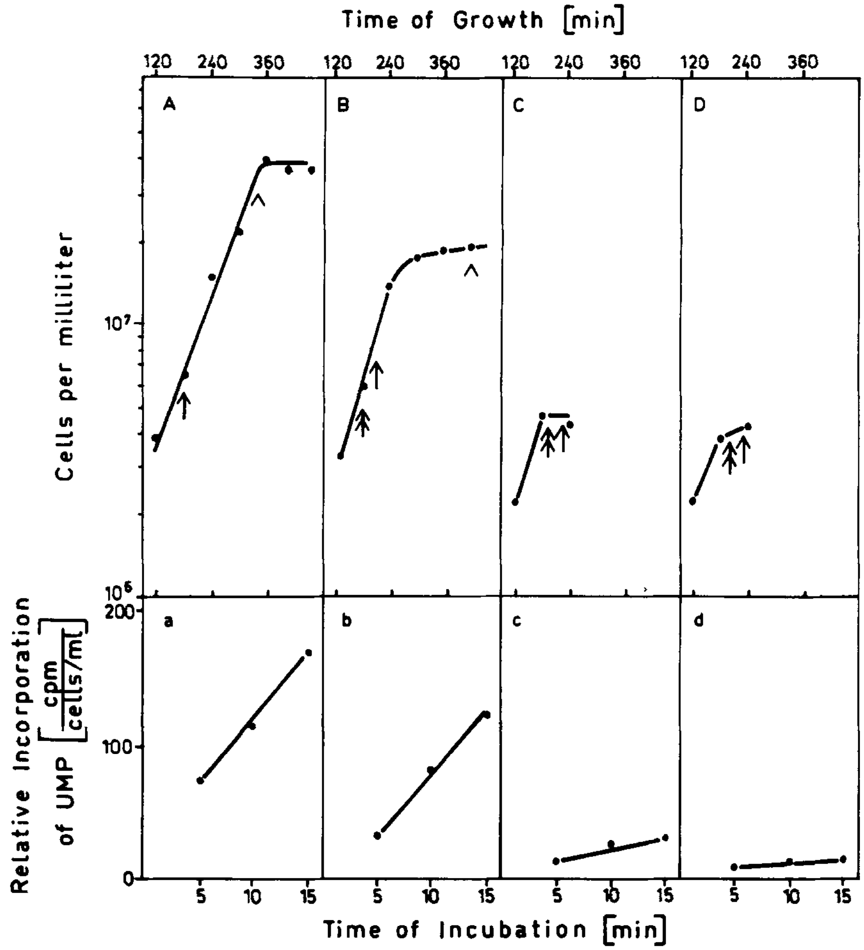

Fig.1 Effects of tyrocidine on growth and RNA synthesis. Cell cultures of Bacillus brevis were grown in a medium containing 5 g casein peptone, 1.5 g yeast extract, 3.5 g NaCl, 3.7 g K_2HPO_4, 3.1 g KH_2PO_4 and 1 g glucose in 1000 ml of water. The experiments were carried out in 100 ml cultures on a rotary shaker at 37°C. Increase in cell numbers was followed with a cell counting chamber and plotted as cells per milliliter. When growth became logarithmic, tyrocidine (SERVA), dissolved in 0.5 ml ethanol, was added to the cultures (↟) under vigorously shaking to a final concentration of 0 (A,a), 5 (B,b), 10 (C,c) and 20 (D,d)/ug per milliliter. Samples of 5 ml were withdrawn as indicated (↑) and incubated with 1/uCi of [^{14}C]uracil (spec.activity 61 mCi per mmol) at 37°C with shaking. After 5, 10 and 15 min duplicate samples of 0.5 ml volume were treated with 1.5 ml of 10% cold trichloroacetic acid. The precipitates were collected on glass fibre filters, washed with 5% trichloroacetic acid and then with ethanol, dried and the radioactivity estimated in an Isocap Nuclear Chicago scintillation counter. The relative incorporation of UMP is plotted as counts per minutes per cells per milliliter. The onset of sporulation was observed by microscopy and occured when indicated (^).

evidence here that tyrocidine inhibits RNA synthesis and leads to a cessation of growth. Gramicidin in comparable molar concentrations does not inhibit RNA synthesis in vivo, and the cells continue to grow. However, sporulation is inhibited by gramicidin. Furthermore, gramicidin can prevent the inhibitory effect of tyrocidine on RNA synthesis.

METHODS AND RESULTS

Tyrocidine effects: To early exponential growing cell cultures of *Bacillus brevis* (ATCC 8185) 5, 10 and 20 μg of tyrocidine per milliliter were added and cell numbers were monitored by optical density measurements and cell counting. It was found that 10 or 20 μg per milliliter of tyrocidine leads to a complete inhibition of growth (Fig.1, C,D). Sporulating cells monitored by microscopy were not observed. The amount of 5 μg per milliliter of tyrocidine leads to some inhibition of growth and also to a delay in sporulation (Fig.1 B) as compared to the untreated control (Fig.1 A). Thirty minutes after adding tyrocidine to the cultures 5 ml samples were withdrawn and incubated with 1 μCi of $[^{14}C]$ uracil. After 5, 10 and 15 minutes 0.5 ml samples of the radioactively labelled cells were tested for RNA synthesis by precipitation with trichloroacetic acid. The results show that RNA synthesis is hardly if at all affected by 5 μg per milliliter of tyrocidine (Fig.1b), but is inhibited almost completely after the addition of 10 or 20 μg (Fig.1 c,d). The control experiment without tyrocidine is presented in Fig.1a.

Gramicidin effects: To early exponential growing cell cultures 10, 30 and 60 μg per milliliter of gramicidin were added. The cells continued to grow (Fig. 2 B,C) even at a concentration up to 60 μg per milliliter (Fig.2 D). At a concentration of 10 and 30 μg milliliter some sporulation took place although it started 5 h later than in the control experiment (Fig.2 A). The results, summarized in Tab.1, demonstrate that sporulation is, however, suppressed in a concentration-fashion, and even waiting 20 h after inoculation did not reveal any spore formation over that seen after 8.5 h. No free spores were visible during that time. To test the RNA synthesis 5 ml samples were withdrawn 30 min

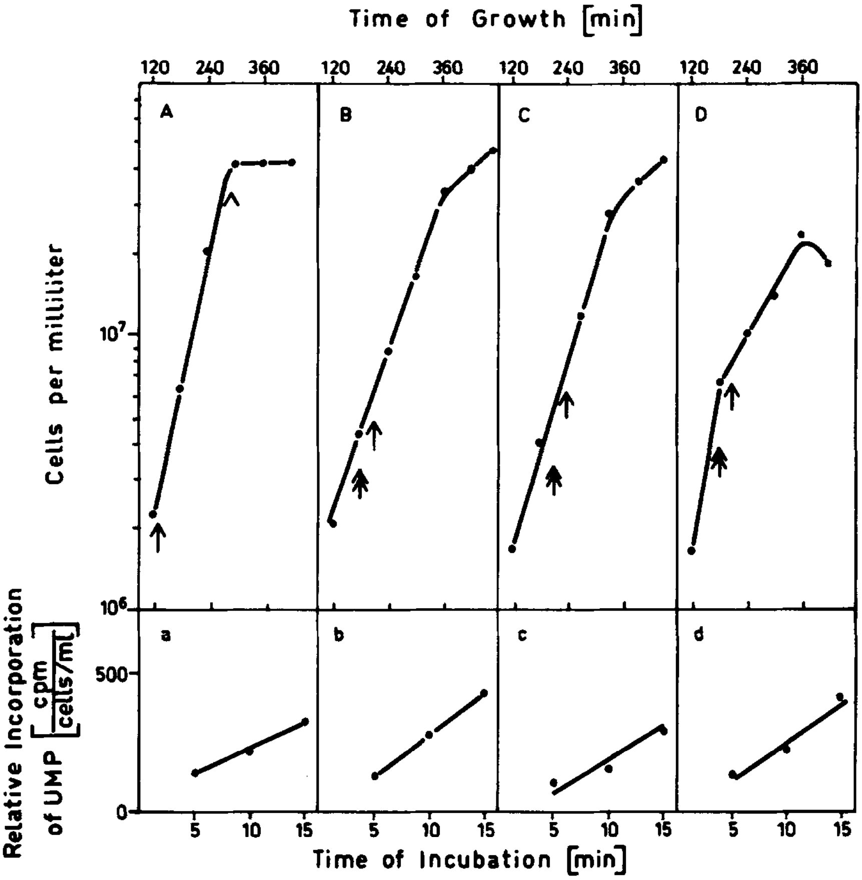

Fig.2 Effects of gramicidin on growth and RNA synthesis. Cell cultures of Bacillus brevis were grown under the conditions described in Fig.1. When they began to grow exponentially gramicidin D (SERVA), dissolved in 0.5 ml of ethanol, was added to the cultures (↟) to a final concentration of 0 (A,a), 10 (B,b), 30 (C,c) and 60 (D,d) μg per milliliter. Samples were tested (↑) for ^{14}C uracil incorporation as in Fig.1. The onset of spore formation is indicated (^).

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after the addition of gramicidin and incubated with 1 μCi $[^{14}C]$ uracil. Samples of 0.5 ml were tested for effects of gramicidin on RNA synthesis by precipitation with trichloroacetic acid. Fig.2 a-d demonstrates that RNA synthesis is not affected by gramicidin at any concentration tested.

Gramicidin effects on tyrocidine actions: Various amounts of a mixture of 70% tyrocidine and 30% gramicidin were added to cell cultures growing exponentially. This mixture corresponds to the ratio of the two peptides as isolated from

Table I Gramicidin effect on sporulation

Gramicidin (/ug per ml)	Sporulating cells (%) after 5 h	8.5 h	20 h
0	95-100	95-100	95-100
10	0	30	30
30	0	10	10
60	0	0	0

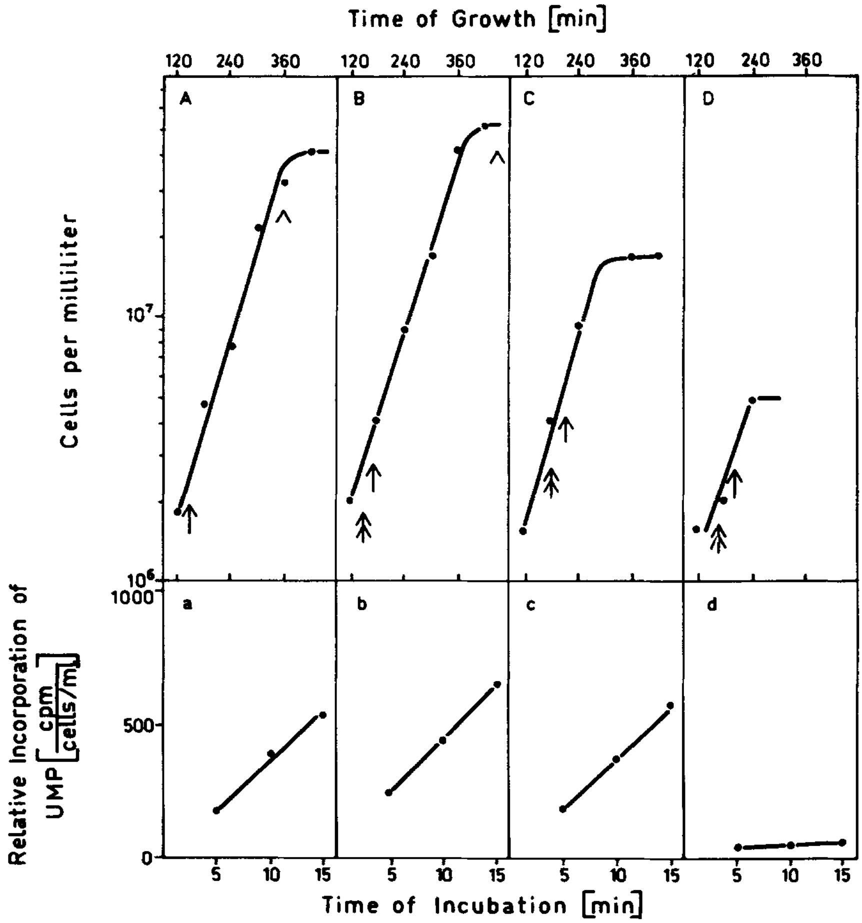

Fig.3 Effects of a mixture of tyrocidine and gramicidin on growth and RNA synthesis. Cell cultures of Bacillus brevis were grown under the conditions described in Fig.1. The antibiotics added (↟) were 0 (A,a), 5/ug tyrocidine plus 2/ug gramicidin (B,b), 10/ug tyrocidine plus 4/ug gramicidin (C,c), and 20/ug tyrocidine plus 8/ug gramicidin (D,d) per milliliter. Samples were tested (↑) for ^{14}C uracil incorporation as described in Fig.1. The onset of spore formation is indicated (^).

Table 2 The effects of mixtures of tyrocidine and gramicidin on RNA synthesis

Antibiotic added (µg per ml) Tyrocidine	Gramicidin	Relative incorporation of UMP
10	-	1.0
10	4	2.7
10	8	2.9
10	12	0.2
-	-	6.6

sporulating cells of Bacillus brevis. The addition of 5 µg per milliliter of tyrocidine in the presence of 2 µg gramicidin had no inhibitory effect on the growth (Fig.3 B) although that concentration of tyrocidine leads to some inhibition when used alone (Fig.1 B). Some growth inhibition was found with 10 µg of tyrocidine plus 4 µg of gramicidin per milliliter (Fig.3 C), however, 10 µg per milliliter of tyrocidine suffices to inhibit growth completely when used alone (Fig.1 C). Even at a concentration of 20 µg of tyrocidine per milliliter cells continue to grow for a certain time when 8 µg of gramicidin are present (Fig.3 D). Sporulation was only detected at a concentration of 5 µg tyrocidine plus 2 µg gramicidin per milliliter and was delayed (Fig.3 B) compared with the control (Fig.3 A). 30 min after addition of the peptide mixtures samples of 5 ml were incubated with 1 µCi of $[^{14}C]$ uracil. After incubation times of 5, 10 and 15 min samples of 0.5 ml were tested for RNA synthesis by trichloroacetic acid precipitation. As shown in Fig.3 c RNA synthesis is not affected by 10 µg of tyrocidine in the presence of gramicidin whereas RNA synthesis is completely inhibited by tyrocidine at this concentration alone (Fig.1 c). However, 20 µg per milliliter of tyrocidine inhibits the RNA synthesis completely even in the presence of gramicidin (Fig.3 d). These experiments clearly indicate that the inhibition of RNA synthesis by tyrocidine is, at least partly, abolished in the presence of gramicidin.

In a different experiment increasing amounts of gramicidin were added to cultures whose growth should have been stopped by the addition of 10 μg per milliliter of tyrocidine. One hour later 5 ml samples were incubated with 1 μCi of $[^{14}C]$ uracil for 15 min. Aliquots of 0.5 ml were then precipitated with trichloroacetic acid and tested for RNA synthesis. As shown in Tab.2 the addition of 4 μg or 8 μg per milliliter of gramicidin led to 3 times higher uracil incorporation than in the control treated with tyrocidine alone. At higher gramicidin concentrations RNA synthesis was once more inhibited.

DISCUSSION

The results reported here demonstrate that the two peptides tyrocidine and the linear gramicidin, which can be isolated from Bacillus brevis, have different effects on growing cells: tyrocidine inhibits RNA synthesis and this inhibition can, at least partly, be revised by gramicidin. This agrees with the results of in vitro experiments (8,9), with one exception: gramicidin inhibits RNA synthesis in vitro but not in vivo. SARKAR and PAULUS recently claimed (5) that RNA synthesis of cells at 15 times the concentration we used is inhibited by tyrocidine at 10 times our concentration. Since our in vitro experiments indicated that the inhibition of RNA synthesis is determined by the relative concentrations of DNA and tyrocidine (8), the results by SARKAR and PAULUS are in essential agreement with our presented here.

SARKAR and PAULUS (5) also demonstrated that when RNA synthesis of cells was inhibited by tyrocidine, DNA synthesis continued indicating that the primary target of the antibiotic is RNA synthesis. Tyrocidine must act therefore within the cells rather than non specifically on the cell membrane. Further supports that the two antibiotics, tyrocidine and gramicidin, penetrate the cell membrane and act within the cells are: 1. Gramicidin affects sporulation, but not net RNA synthesis. 2. Radioactively labelled tyrocidine as well as gramicidin are found in the cell pellet after cells have been incubated with these antibiotics and extensively washed thereafter. 3. The relative radioactivity found in the cell pellet (cpm per cell density) was dependent on the state of the cell culture. Early vegetative growing cells were found to be highly labelled whereas cells already synthesizing the antibiotics were poorly labelled (Ristow and Schazschneider, unpublished).

YOSHIDA et al. (3) found in 1966 that young cultures of *Streptomyces antibioticus* are inhibited in their growth by the addition of actinomycin D whereas cultures synthesizing already this antibiotic are insensitive to exogenous drug. It seems reasonable to assume that this was due to an inhibition of RNA synthesis and might reflect a similar inhibitory mechanism as in our system.

In contrast to the results of JAYARAMAN and KANNAN (10) we only observed sporulation after the addition of low concentrations of both, tyrocidine and gramicidin, to growing cell cultures, and at higher concentrations, no sporulation was observed. This might be due to critical concentrations of the peptides and different growing conditions these authors used.

Our result that gramicidin does not inhibit net RNA synthesis *in vivo* but does affect sporulation will be a matter of further investigations.

In conclusion the possibility raised by our previous *in vitro* results that they might reflect *in vivo* interactions of tyrocidine, gramicidin and RNA synthesis has been confirmed by the experiments presented here.

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