SELECTIVE INHIBITION OF SPORULATION OF BACILLUS SUBTILIS BY NETROPSIN

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SUMMARY. At low concentrations, the basic-polypeptide antibiotic, netropsin, did not inhibit growth, over-all RNA synthesis, replication of phage ϕe, or synthesis of some catabolite-repressed enzymes in Bacillus subtilis 168. Cells developed normally until t_2 of sporulation, but no refractile spores were formed in the presence of the antibiotic. The selective inhibition of sporulation by netropsin may be related to the base composition or sequence of some sporulation specific genes.

INTRODUCTION. Analysis of the initial events of sporulation in Bacilli is complicated because initiation occurs at the end of the exponential phase of growth when many enzymes are released from catabolite repression (1). It is difficult to determine whether the enzymatic activities which increase at this time are really essential for sporulation. Studies have shown that release from catabolite repression is necessary, but not sufficient for initiating sporulation (2). It would be advantageous for the understanding of initiation to have an inhibitor which would permit growth and catabolite derepression to occur normally, but which would specifically inhibit sporulation processes.

In our current studies we have found that netropsin, a basic polypeptide antibiotic produced by Streptomyces netropsis (3), does not inhibit normal growth or release from catabolite repression in Bacillus subtilis 168, but does inhibit sporulation selectively. The mode of action of this antibiotic has been reported to be at the level of transcription and replication since it has a high affinity for adenine-thymine (A-T) enriched sequences of DNA (4). The use of this antibiotic should lead to a better understanding

of the control mechanisms involved in the regulation of catabolite repressed enzymes and sporulation specific genes.

MATERIALS AND METHODS

Organism and media: B. subtilis 168 wild type was grown at 37 C in a modified Schaeffer medium (2 x SG) (5) for all experiments.

Netropsin treatment: An overnight culture of B. subtilis was diluted and transferred to 20 ml of 2 x SG medium at 37 C in a Klett flask. Growth was followed with a Klett-Summerson Colorimeter. At a Klett reading of 110 units, the cells were transferred to fresh medium equilibrated at 37 C. When the cultures attained 40 units, the cultures were placed into Klett flasks containing different amounts of netropsin. Microscopically distinct stages of sporulation were determined with a phase-contrast microscope.

Effect of netropsin added at various times: Synchronized cells were subcultured in medium free of netropsin. At selected periods of growth 10 ml aliquots were removed from the parent flask and placed into Klett flasks containing 10 µg of netropsin until t_{24}. The cultures were examined for their Klett reading, pH, and spore formation.

Extracellular protease assay: Extracellular protease activity in the growth medium was assayed as reported previously (6).

^{3}H-Uridine pulse-labelling of RNA: The incorporation of ^{3}H-uridine into RNA was measured by the method described previously (7).

RESULTS

Growth and sporulation of B. subtilis in netropsin medium: The effect of netropsin on growth and sporulation was tested by culturing cells at various concentrations of the antibiotic (Fig. 1).

The growth rate of B. subtilis 168 in 2 x SG medium with netropsin was the same as that of cells growing without netropsin if the concentration of the antibiotic did not exceed 2 µg/ml. Greater concentrations of netropsin inhibited both the growth rate and the final number of cells. Control cultures normally had 70% refractile sporangia by t_6 (t_n = n hours after the end of exponential growth) and greater than 90% by t_8 and t_{24}. The netropsin-treated cells lost the ability to form refractile bodies when they were grown in 2 x SG with concentrations of inhibitor greater than 0.5 µg/ml. To determine the period at which netropsin could inhibit spore formation the antibiotic was added to cultures at various times during sporulation and checked for refractile spore formation at t_{24}. The cells were unable to sporulate even if the addi-

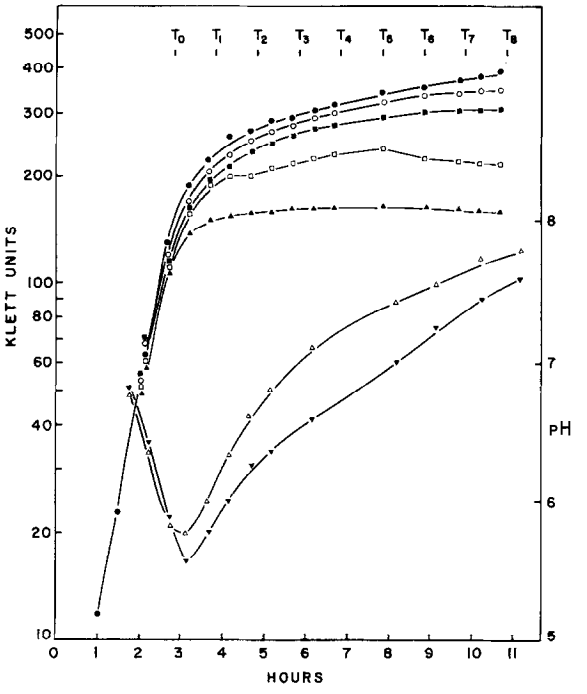

Fig. 1 Effect of netropsin on growth and sporulation of B. subtilis Cells were grown in 2 x SG medium (5) with 0 (●), 1 (○), 2 (■), 4 (□), and 10 (▲) µg per ml of netropsin at 37 C with vigorous shaking. The pH was also measured for the control (△) culture (no netropsin) and the netropsin-containing (1 µg/ml) culture (▼). Netropsin was added to the cultures at 1.5 hrs. T_o represents the end of the exponential phase of growth and T_n is the hourly periods after T_o.

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tion of the drug was delayed to t_4. Netropsin added after t_4 allowed some spore formation, probably due to asynchrony of sporulation, since these cultures showed mixtures of free spores and vegetative cells at t_{24}.

Normal production of pyruvate and acetate (8) and their later utilization was indicated by the parallel pH changes observed in cultures with and without the drug (Fig. 1). The change in pH from below 7.0 to above 8.0 was observed in cultures treated with netropsin either early or late in the exponential phase of growth. Concentrations of the antibiotic greater than 5 µg/ml significantly decreased the growth rate and the rate of accumulation of the acidic compounds. However, the pH in these cultures was seen to rise slightly after their growth was completely stopped.

One possibility for the inhibition of sporulation but not of growth by netropsin was a greater permeability of sporulating cells to netropsin. To check this, cells grown to t_2 in the presence of netropsin (1 μg/ml) were diluted into fresh medium. One flask contained netropsin (1 μg/ml) and the other did not. The cells in both flasks grew without a lag phase and at the same exponential rate. Thus the effect of netropsin on sporulation did not affect the growth properties of the cells. The culture containing netropsin was grown to t_2 and the cells at this stage had all the properties associated with t_2 cells, i.e. small, highly motile cells.

Bacteriophage development in netropsin grown cells: Since previous studies (9) had indicated that netropsin could inhibit phage production, cells were infected with phage Øe in the presence of netropsin at the concentration which allowed growth, but inhibited sporulation. Control cells infected at a MOI of 0.05 in early log phase of growth with Øe were seen to lyse about 2.5 hr later. The same cells grown and infected in the presence of netropsin (1 μg/ml) lysed at the same time. It was further shown that Øe phage could effectively infect and lyse sporulation-inhibited cells obtained at t_2 through t_{24}.

Protease production in netropsin grown cells: One of the early functions associated with sporulation is the production of proteases (10). The effect of netropsin on protease induction was therefore investigated. *B. subtilis* grown in the absence of netropsin were spread onto plates of A-K (Difco) agar containing 1% dry milk with and without netropsin (1 μg/ml). The zones of hydrolysis in the drug-containing plates at 24 hr had the same diameters as those plates without netropsin. The colonies on the netropsin plates were larger than the controls. By 48 hr, the netropsin-treated plates had colonies devoid of spores that were very much larger than those on the control plates which were mainly free spores.

Cell-free culture media obtained at t_4 showed the presence of proteolytic activity. The netropsin-treated cells elaborated about half the amount of

total proteases of the control cultures, but showed the same percentage of metal-requiring and serine proteases as did the controls.

^{3}H-Uridine pulse-labelling of netropsin grown cells: Since netropsin has been shown to be an inhibitor of RNA polymerase, the ability of netropsin-containing cultures to synthesize RNA was tested.

The incorporation of ^{3}H-UMP into RNA by cells grown in the presence of 1 µg/ml of netropsin indicated that the overall rate of RNA synthesis was similar to that of untreated cultures (Fig. 2). The pattern of RNA synthesis was similar to that reported previously (7).

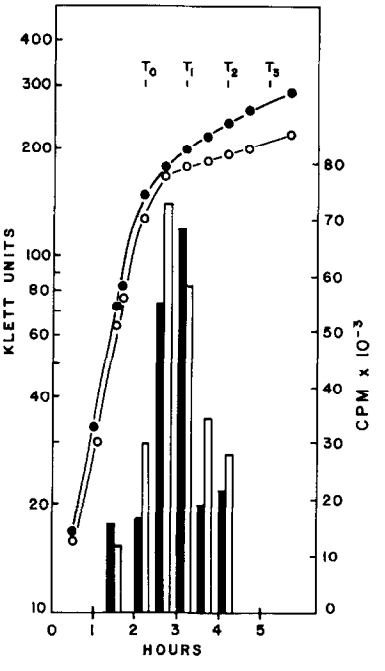

Fig. 2 RNA synthesis in the presence and absence of netropsin. Cells were grown in the presence (open circle) of 1 µg/ml of netropsin and the absence (dark circle) of the antibiotic in 2 x SG medium. One ml aliquots were taken from the master cultures and mixed with ^{3}H-uridine (1 µC; 27 Ci/mmole) for 1 minute with vigorous shaking at 37 C. The incorporation was stopped by adding 1 ml of ice cold 10% trichloroacetic containing 500 µg of unlabeled uridine. RNA synthesis in the absence and presence of netropsin is represented by black and white bars, respectively.

DISCUSSION. In a selected range of concentrations, the basic oligopeptide antibiotic, netropsin, showed no deleterious effects on the vegetative growth of B. subtilis. The ability of these cells to undergo sporulation was inhibited, even though several of the early sporulation events such as protease and the utilization of pyruvate and acetate were expressed. Netropsin did not appear to alter the ability of the cells to complete their morphological changes up to t_2, to produce proteolytic enzymes, to utilize acidic compounds, to synthesize RNA, to support bacteriophage development, or to grow when subcultured into fresh medium with or without netropsin. These observations suggested that some sporulation specific events were sensitive to the drug.

It has been shown that DNA and RNA synthesis can be inhibited by the association of netropsin with the template (4). That over-all RNA synthesis was not affected at low concentrations of antibiotic was shown by the growth of cells, by the ability of Øe to complete its lytic cycle, and by the pulse-labelling studies. The normal development of netropsin-treated cells to t_2 showed that DNA synthesis during log phase or post-exponential phase was also not affected. The final cell division between t_0 and t_2 with its concommitant DNA synthesis has been shown by Leighton et al. (11) to be necessary for sporulation to occur.

Netropsin inhibits the derepression of catabolite-repressed enzymes in E. coli if added prior to their induction (12). Proteases and tricarboxylic acid cycle enzymes are produced early in sporulation (10) and these catabolite-repressed enzymes were not sensitive to netropsin at selected concentrations. These observations support the idea expressed previously that release from catabolite repression is necessary but not sufficient for sporulation (2).

Netropsin is known to bind specifically to A-T pairs and not to G-C pairs (4). One possible explanation for our results is that the promoter sites for vegetative and catabolite repressed genes may not be as enriched in A-T pairs as some sporulation-specific genes thus resulting in selective inhibition of the transcription of those by netropsin. Rogolsky and Nakamura (13) have

reported the preferential inhibition of sporulation by ethidium bromide. Their data also suggest that the DNA composition of sporulation specific genes may be different from that for log phase genes.

The use of netropsin should allow a finer dissection of the early sporulation events and may permit us to separate sporulation-specific events from log phase and catabolite repressed functions. Further experiments are obviously required to explain the selective inhibition by netropsin.

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