# INTRACELLULAR RIBONUCLEASE ACTIVITY IN STATIONARY PHASE CELLS OF BACILLUS SUBTILIS

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## SUMMARY

A RNase was isolated and partially purified from stationary phase cells of Bacillus subtilis. The activity of this RNase is stimulated by $Mn^{2+}$ and inhibited by $Ca^{2+}$. The activity of the enzyme reaches a maximum at about 2-3 h after the end of exponential growth ($t_2$-$t_3$). The RNase is able to degrade, in addition to other polyribonucleotides, the RNA synthesized in vitro by the RNA polymerase on φe DNA template but it is inactive on the poly-AU, product synthesized on poly-d(AT) template.

In pursuing one aspect of our investigation of the biochemical events associated with spore morphogenesis in Bacillus subtilis, we focussed our attention on nuclease activity during the sporulation process. A particular reason to examine this problem was to assess the possibility of interference by nuclease activity in those in vitro assays of transcription of various template DNA's which utilise partially purified RNA polymerase prepared from vegetative and sporulating cells.

In the present communication we report the isolation, from B. subtilis sporulating cells, of manganese-stimulated ribonuclease (RNase), capable of hydrolyzing, in addition to other polyribonucleotides, the RNA synthesized in vitro by the RNA polymerase on phage φe template DNA, but which is inactive towards the product synthesized on poly-d(AT) template (poly-AU). This ribonuclease reaches a maximum of activity about 2-3 hours after exponential growth has stopped ($t_2$-$t_3$)

## MATERIALS AND METHODS

Bacillus subtilis, 168M ($trp^-$) and its asporogenic derivative 12A (1) were grown on nutrient broth (Difco) medium as described (2).

For the synthesis of RNA in vitro, the RNA polymerase from vegetative cells of B. subtilis was purified according to Shorenstein et al. (3). The enzyme fraction IV was used with a specific activity of 720 units/mg of protein in the assay conditions described by Orrego et al. (4). Sigma factor and core enzyme were obtained by following the purification procedure through the phosphocellulose chromatography step. For the isolation of larger amounts of RNA or poly-AU the ingredients and volume of the respective assay mixtures (4) were multiplied by a factor of 5 or 8. The products were then purified by phenol extraction and ethanol precipitation as described by Travers (5).

RNase activity was measured by the rate of degradation of labeled polyribonucleotides into acid-soluble material. The incubation mixture contained, in 0.25 ml : 0.04 M tris-HCl buffer, pH 7.9 ; 0.01 mM dithiotreitol (DTT) ; 0.4 mM K-phosphate ; 1 to 2 µg of $^3$H-RNA and 5 to 10 µg of enzyme protein. If not otherwise stated, the reaction mixture was incubated 10 min at 34°C, the reaction was stopped by the addition of 1 ml of 10 % TCA and 100 µl of 0.1 M Na-pyrophosphate, and the mixture was kept on ice for an additional 30 min. The TCA precipitable material was filtered on Whatman GF/C filters, washed extensively with 2.5 % TCA, and then with 1-2 ml of ethanol, and dried. The radioactivity was counted in a Intertechnique scintillation counter. One unit of RNase activity corresponds to the degradation of 1 µg of ribonucleotide per 10 min under the conditions described. Protein concentrations were estimated by the method of Lowry et al. (6) with cristalline bovine albumin as standard.

Buffer A : 10 mM tris-HCl, pH 7.9 ; 10 mM $MgCl_2$ ; 10 mM KCl ; 0.3 mM DTT ; 0.1 mM EDTA. Buffer B : 10 mM K-phosphate, pH 7.3 ; 20 mM KCl ; 0.3 mM DTT ; 5 % glycerol. Buffer C : 10 mM tris-HCl, pH 7.9 ; 10 mM $MgCl_2$ ; 10 mM KCl ; 0.3 mM DTT ; 50 % glycerol.

## RESULTS

It was generally observed that the capacity to transcribe in vitro the phage φe DNA (or B. subtilis DNA) sharply decreases when the RNA polymerase is isolated from B. subtilis sporulating cells instead of using the enzyme from exponentially grown cells (4, 7, 8, 9). No such decrease is observed with poly-d(AT) as template. Although it was suggested that the decrease in the transcription capacity of the sporulating RNA polymerase might be due to a loss of the sigma factor activity, no conclusive evidence in support of this view has been reported (10). Therefore, the possibility cannot be excluded that the presence of an inhibitor, associated to the RNA polymerase in sporulating cells, might cause the observed decrease in the transcription capacity. This idea was suggested by the results shown in Table 1. It can be seen that the drop in transcription capacity (reflected by the ratio φe DNA/poly-d(AT)), observed with the partially purified RNA polymerase from sporulating cells ($t_4$ and $t_8$), is abolished with the enzyme isolated from dormant spores, which show values similar to those obtained with the exponential cell enzyme. This would suggest that the partially purified RNA polymerase from sporulating cells contains an inhibitor which is absent in the enzyme from dormant spores.

These observations prompted us to search for an inhibitor of φe DNA transcription in sporulating cells. We first observed that, by adding a small amount (60 µg of protein) of an ammonium sulfate fraction, prepared from sporulating cells of B. subtilis ($t_3$ cells), to an in vitro RNA synthesizing system catalyzed by the RNA polymerase purified from vegetative cells of this organism (see Materials and Methods), a drop of about 30 % in the specific activity of the enzyme was obtained, as com-

TABLE 1. B. subtilis RNA polymerase activity from vegetative and sporulating cells and from dormant spores.

| Source of enzyme | | Purification step | Specific activity | | |
|---|---|---|---|---|---|
| | | | φe DNA | poly-d(AT) | φe DNA / poly-d(AT) |
| Vegetative cells | | $(NH_4)_2SO_2$ | 33 | 5 | 6.6 |
| | | DEAE-cellulose | 26.3 | 5.7 | 4.6 |
| Sporulating cells | $t_4$ | $(NH_4)_2SO_4$ | 8 | 20 | 0.4 |
| | $t_8$ | $(NH_4)_2SO_4$ | 8 | 18 | 0.44 |
| Dormant spores | | $(NH_4)_2SO_4$ | 1.3 | 1 | 1.3 |
| | | DEAE-cellulose | 16 | 1.7 | 9.5 |
| | | glycerol gradient | 290 | 43 | 6.8 |

pared to a control not containing the sporulating extract (see later in Table 5). We further found that the inhibition produced by the $t_3$-extract is a non dialysable protein, resistant to heat (10 min at 100°C), and finally, that it contained a ribonuclease activity. This activity was then purified 25 fold from a stationary phase culture of B. subtilis 168M, harvested between $t_2$-$t_3$, and washed and disrupted as previously described (4). After removal of cell debris, the supernatant fluid was freed of ribosomes, by centrifugation for 90 min at 100,000 g in a Spinco ultracentrifuge, and fractionated with ammonium sulfate. The protein fraction precipitating between 42-60 % saturation was collected by centrifugation at 20,000 g for 15 min (step 1). The pellet was dissolved in a small volume of buffer A, dialysed against the same buffer, and chromatographed on DEAE-cellulose. The RNase activity was eluted in a broad peak between 0.07-0.15 M KCl (step 2). A minor peak of activity was also eluted at 0.23 M KCl, which corresponds to the peak of RNA polymerase activity. This peak was left out of the RNase preparation. The main peak fractions were pooled, brought to 65 % saturation with ammonium sulfate, and centrifuged after stirring for 30 min at 4°C. The pellet was dissolved in

TABLE 2. Substrate specificity of B. subtilis RNase

| Substrate | Specific activity (units/mg protein) |
|---|---|
| $^{3}$H-RNA(1) | 127 |
| $^{3}$H-RNA(2) | 30 |
| $^{3}$H-poly-AU(3) | 0 |
| $^{14}$C-poly-G | 156 |
| $^{14}$C-poly-C | 642 |

(1) RNA synthesized in vitro by B. subtilis RNA polymerase on phage φe DNA template.

(2) Bulk RNA isolated from a B. subtilis exponential culture labeled with $^{3}$H-uridine.

(3) synthesized in vitro by B. subtilis RNA polymerase on a synthetic poly-d(AT) template.

a small volume of buffer B, dialysed 15 h against the same buffer, and applied on a 2 ml hydroxyapatite column equilibrated with the same buffer. Under these conditions the RNase activity is not retained on the column and was found in the effluent. The RNase was then concentrated by ammonium sulfate precipitation (65 % saturation), dissolved in buffer C, dialysed against the same buffer, and stored at -20°C (step 3).

Table 2 shows the substrate specificity of the RNase. It is interesting to note that the RNA synthesized in vitro by B. subtilis RNA polymerase in the presence of phage φe template DNA is degraded by the RNase, whereas the poly-AU synthesized on poly-d(AT) template is not hydrolysed. Figure 1 shows the kinetics of action of the RNase on these two substrates. Furthermore, the apparent specific activity of the RNase when bulk B. subtilis RNA is used as substrate is low compared to that obtained with the other degradable substrates. For greater insight into the specificity of the RNase we are currently analysing the products of hydrolysis of various substrates.

After heating 10 min at 100°C, the enzyme is still able to degrade B. subtilis DNA (assayed with $^{32}$P-labeled DNA). But at the present stage of purification it cannot be decided if the hydrolysis of DNA and RNA is due to the same protein or, as it seems most likely, results from the action of a heat stable DNase similar to that found by McCarthy and Nester in exponential cells of B. subtilis (11).

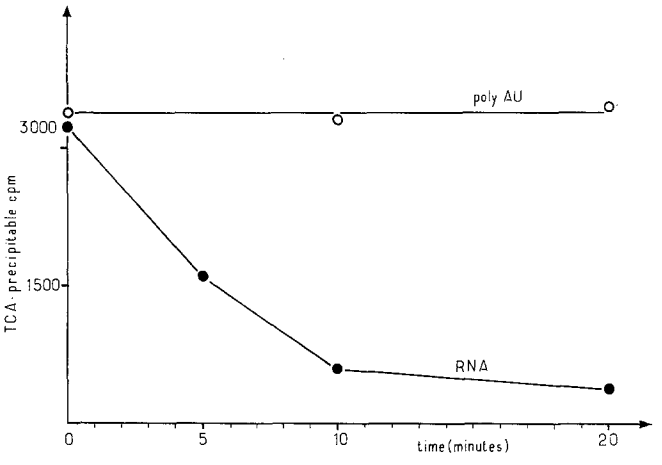


Figure 1. Kinetics of action of RNase on RNA and poly-AU. RNA and poly-AU were synthesized in vitro by B. subtilis RNA polymerase respectively in presence of phage φe DNA and synthetic poly-d(AT) used as templates.

The RNase has a maximum of activity at pH 7.9 ; it differs therefore from the intracellular RNase, isolated by Nishimura and Maruo (12) from B. subtilis, which has an optimum pH at 5.8 and which is inactivated by heating at 100°C for 5 min. No phosphodiesterase activity (assayed by p-nitrophenylphosphate) could be detected in the partially purified RNase preparation. The RNase activity is not derived from polynucleotide phosphorylase, since this enzyme is completely inhibited by phosphate ions under the assay conditions employed.

Table 3 shows the effect of divalent cations on RNase activity. It can be seen that $MnCl_2$ produces about a twenty five fold stimulation in the specific activity. This stimulation is completely abolished by EDTA. The addition of $MgCl_2$ produces relatively little stimulation. $CaCl_2$ produces an inhibition which can be reversed by the addition of EGTA.

It is shown in Table 4 that the RNase activity is low in exponential cells, increases at the beginning of stationary phase, and reaches a maximum at about $t_2$-$t_3$. A similar pattern of activity was found in a zero stage sporulation mutant (12A).

Table 5 shows the effect of an extract prepared from sporulating cells harvested at about $t_3$, on RNA synthesis catalyzed by either vegetative RNA polymerase-holoenzyme, core enzyme, or core plus σ. It can be seen that the inhibition is exerted only when active sigma factor is present together with the core. The addition of $t_3$-extract to the core

TABLE 3. Effect of cations on RNase activity

| Additions | Specific activity (units/mg protein) |
|---|---|
| None | 5.2 |
| $MgCl_2$ (10 mM) | 11.8 |
| $MnCl_2$ (5 mM) | 127 |
| $MnCl_2$ (5 mM) + EDTA (20 mM) | 7.2 |
| $CaCl_2$ (0.1 mM) | 2.4 |
| $CaCl_2$ (0.1 mM) + EGTA (10 mM) | 5.74 |

The assay conditions were as described in Materials and Methods.

TABLE 4. Kinetics of appearance of RNase activity

| Time of harvesting cells | Specific activity |
|---|---|
| $t_{-1}$ | 0.53 |
| $t_{0.5}$ | 1.53 |
| $t_2$ | 3.25 |
| $t_3$ | 1.2 |
| $t_5$ | 1.5 |

Activity was measured in ammonium sulfate enzyme fractions in presence of $MgCl_2$, i.e. under conditions of RNA synthesis in vitro (see Materials and Methods).

enzyme alone has no effect. However, when the step 3 purified RNase was added to the same incubation mixture no inhibition of RNA synthesis was observed. This would suggest that, in addition to the RNase, the $t_3$ extract also contains an inhibitor acting on the in vitro RNA synthesizing system. It should be kept in mind, however, that this system, with $\phi$e template DNA, always contains $Mg^{++}$ cations (4), which, as seen in Table 3, does not favor maximum activity of the RNase. When $MgCl_2$ is

TABLE 5. Effect of extracts prepared from sporulating cells harvested at $t_3$, on RNA synthesizing system in presence of B. subtilis vegetative RNA polymerase.

| Vegetative RNA polymerase | Addition | Specific activity (units/mg of protein) |
|---|---|---|
| Holoenzyme | - | 790 |
| Holoenzyme | $t_3$-extract[1)] | 560 |
| Core enzyme | - | 73 |
| Core enzyme | $t_3$-extract | 70 |
| Core enzyme + σ | - | 1014 |
| Core enzyme + σ | $t_3$-extract | 565 |
| Holoenzyme | $t_3$-RNase[2)] | 785 |

1) $t_3$-extract is an ammonium sulfate fraction (see Materials and Methods) containing 60 µg of protein.

2) $t_3$-RNase is the step 3 purified RNase containing 38 µg of protein.

replaced by $MnCl_2$ (in this incubation mixture), the specific activity of the RNA polymerase is decreased by about 50-60 % ; under these conditions the addition of purified RNase decreases the RNA synthesis by about 60 % (not shown in the Table 5).

## DISCUSSION

Extracellular nucleases from B. subtilis, capable of hydrolyzing DNA and RNA, have been described by a number of investigators (13, 14). Several deoxyribonucleases have also been identified in extracts of B. subtilis (11, 15, 16). Extracellular RNases were obtained from this microorganism which show specificities which are different than that of pancreatic RNase and $T_1$ RNase (17, 18). Nishimura and Maruo (12) and Yamasaki and Arima (19) have reported the existence, in extracts of B. subtilis, of an intracellular ribonuclease which is distinct in its properties from the extracellular RNases.

The intracellular RNase described here differs from that studied by the above authors by its optimum pH, heat stability and insensitivity to nucleosides triphosphates. It should be emphasized that the enzyme (or enzyme complex) described here is also capable of degrading B. sub-

tilis DNA, but there is no evidence that the two activities reside in a single protein. Further studies on more highly purified enzyme could solve this problem.

The fact that this RNase is able to degrade the RNA, synthesized in vitro by B. subtilis RNA polymerase in the presence of φe template DNA, but not the poly-AU product synthesized on a poly-d(AT) template, is of particular interest. It may, indeed, explain, at least in great part, the loss of transcription capacity of the φe DNA generally observed when partially purified RNA polymerase derived from B. subtilis sporulating cells is used. The results shown in Table 5 strongly support the view that the so-called "change in template specificity", observed in vitro when φe DNA and a partially purified RNA polymerase isolated from sporulating cells are used (7, 8, 20), might in fact be the result of action of an RNase-containing inhibitory complex present in the sporulating cells.

It is important to emphasize that, in recent experiments, Kawamura and Ito (21) have clearly demonstrated that, in vivo also, the transcription of phage φe genes takes place even during the late stages of sporulation. Thus the RNA polymerase in B. subtilis sporulating cells is able to transcribe φe DNA in vivo and, as shown by Bonamy et al. (22) is also able to transcribe its own DNA, since ribosomal RNA continues to be made during the sporulation process.

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