# SYNTHESIS OF A FACTOR STIMULATING TRANSCRIPTION IN OUTGROWING BACILLUS CEREUS SPORES.

Amikam Cohen and Alex Keynan
Department of Microbiological Chemistry
The Hebrew University - Hadassah Medical School

Received December 29, 1969

## SUMMARY

Evidence is presented which suggests that RNA synthesis in outgrowing B. cereus spores is partially controlled by the synthesis of a protein or proteins which occurs 2-3 minutes following germination.

## INTRODUCTION

Protein synthesis in outgrowing spores has been demonstrated to be sequential and depends on de novo synthesis of messenger RNA.[1] Therefore, the control of RNA synthesis during spore germination and outgrowth has to be of importance in the process of spore differentiation to vegetative cell.

Recently Losick and Sonenshein[2] have reported that RNA polymerase of bacterial cells and spores have different template specificity and suggested that control of transcription in sporulating cells may result from differences in the $\sigma$ component of the enzyme. In this communication we report experimental results suggesting that a protein which partially controls RNA synthesis in outgrowing spores is synthesized within the first minutes following germination. The possible nature of this protein is discussed.

## MATERIALS AND METHODS

B. cereus T spores were a generous gift from Dr. Z. Evenchik. The spores were stored lyophilized and suspended in distilled water before experiments.

All germination and outgrowth experiments were conducted at 30°C. Before initiation of germination, spores were subjected to heat shock at 60° for 40 minutes in 0.1 M phosphate buffer pH 6.8. Unless otherwise indicated, spore concentration during heat shock was 5 mg/ml, and final spore concentration in germination and outgrowth medium - 0.5 mg/ml.

Two media were used in these experiments: CDGS-glucose - a chemically defined growth and sporulation medium[3] was modified by adding 100 µg/ml of Adenosine

and 100 µg/ml L. alanine. Glucose concentration was 5 mg/ml. Germination medium - contains 100 µg/ml L. alanine and 100 µg/ml Adenosine in 0.1 M phosphate pH 7.0. This medium supports only germination and limited protein synthesis.[4]

Incorporation of $^{3}H$ uracil into acid insoluble material was determined by applying samples on paper discs, (Whatman 1 mm 2.3 cm) and subsequent washing of the discs in 10% Trichloroacetic acid (acid) and 95% ethanol. Absorbancy was determined by Beckman Spectrophotometer equipped with Gilford photocell and recorder.

## RESULTS

The effect of germinating spore exudate on rate of RNA synthesis:

Heat shocked spores were incubated in germination medium, samples were taken at time intervals, the germination exudate removed by filtration and the washed spores were resuspended either in fresh medium or in their own filtrates. The rate of incoporation of $^{3}H$ uracil into acid insoluble material by outgrowing spores washed after 8 minutes incubation and resuspended in fresh medium or filtrate is

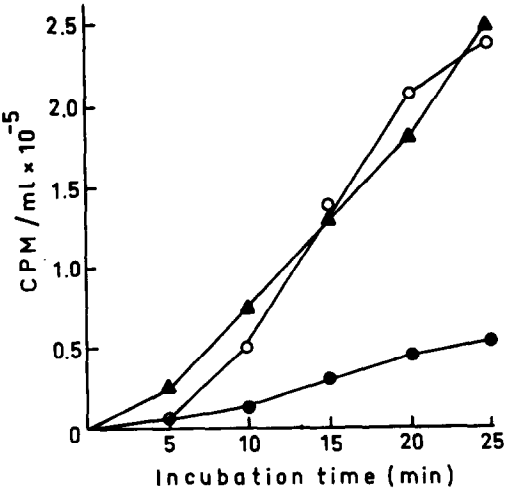


Figure 1

Incorporation of $^{3}H$ uracil into acid insoluble material in the presence and absence of spore exudate. Heat activated spores were incubated in germination medium for 8 minutes, then washed on Millipore filter and resuspended in one of the following: a) Their own filtrates (0 — 0); b) fresh germination medium (● — ●); germination medium supplemented with the following amino acids (mg/ml): L glutamic acid, 1.8; L leucin, 0.8; L. valine, 0.3; L threonine, 0.17; L methionine, 0.07; L histidine, 0.05 (▲ —▲ ). 5 µc of 1 c/mmole $^{3}H$ uracil was added to 1 ml of each of the reaction mixtures and incorporation of radioactivity to acid insoluble material was measured as described in text.

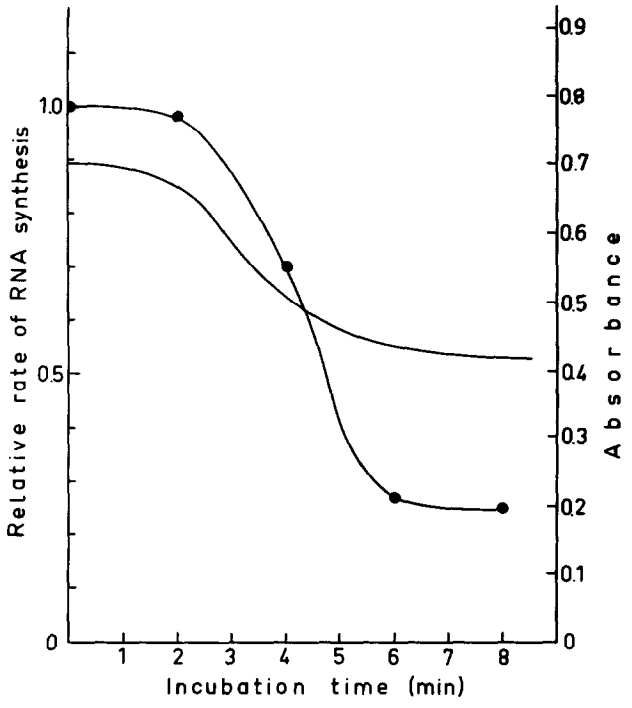


Figure 2

The effect of time of removal of spore exudate of the rate of RNA synthesis during germination and outgrowth. Heat activated spores were incubated in germination medium, at time intervals, samples were taken, the exudate was removed and the spores were washed by filtration. The washed spores were resuspended in fresh germination medium containing 5 μc/ml $^{3}$H uracil and incorporation of radioactivity to acid insoluble material was determined. Solid curve is the absorbancy of the master culture; (● — ●) maximum rate of $^{3}$H uracil incorporation to acid insoluble material relative to the rate of incorporation in spores washed at time 0.

shown in Fig. 1. The relation of the rate of $^{3}$H uracil incorporation into acid insoluble material by washed spores to the time of removal of exudate is illustrated in Fig. 2.

Comparison of these results with the decrease of absorbancy of the same spore suspension indicates that the release of spore components which stimulate RNA synthesis occurs simultaneously with spore germination, as measured by the decrease of absorbancy. Exudate of germinating spores treated with activated charcoal did not restore the rate of RNA synthesis by washed spores, nor did mixtures of nucleotides or nucleosides. On the other hand, addition of a mixture of amino acid fully restored the rate of RNA synthesis.

These experiments suggested the possibility that protein synthesis is required for stimulation of RNA synthesis in outgrowing spores. Therefore, the effect of inhibitors of protein synthesis on this system was tested. Fig. 3 shows the effect of puromycine and chloromphenicol added before initiation of germination on the incorporation of $^{3}H$ uracil into acid insoluble material. Spores germinating in the presence of these protein synthesis inhibitors incorporate $^{3}H$ uracil into acid

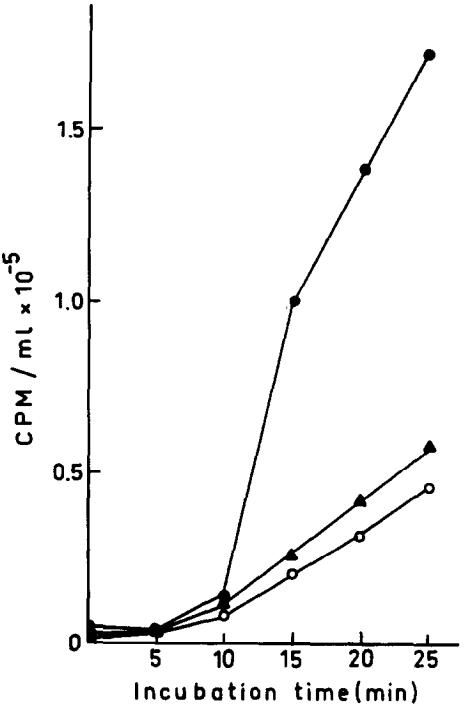


Figure 3

The effect of chloromphenicol and puromycine on RNA synthesis. 100 µg chloromphenicol or 100 µg puromycine were added to 1ml of heat shocked spores in germination medium, which contained 5 µc/ml $^{3}H$ uracil. Incorporation of radioactivity to acid insoluble material was determined. (● — ●) no inhibitor was added. (▲ —▲ ) chloromphenicol was added. (0 — 0) puromycine was added.

---

insoluble material at a significantly lower rate than spores incubated without inhibitor. Unlike germinating spores, B. cereus vegetative cells incubated under similar conditions incorporate uracil into acid insoluble material at similar rates in the presence and absence of puromycine.

If the protein (or proteins) which affect RNA synthesis during germination and outgrowth of spores are synthesized de novo at a specific time during this process, one should be able to detect the time of synthesis of this protein by studying the

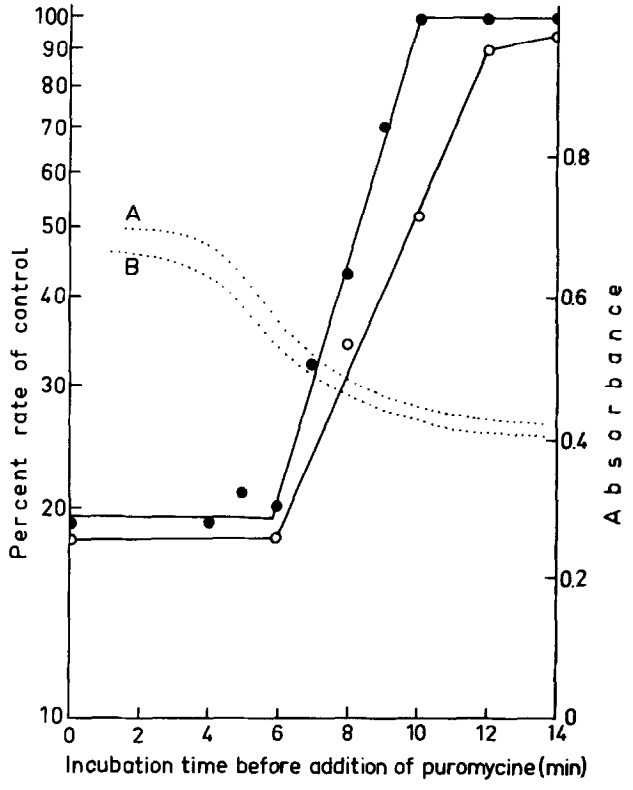


Figure 4

The effect of time of addition of puromycine on the rate of RNA synthesis. Puromycine (100 µg/ml) was added to incubation mixtures in germination medium (0 — 0), or CDGS-glucose (● — ●), at different times following initiation of germination. $^{3}H$ uracil was added 20 min. after initiation to each of the mixtures and rate of incorporation of radioactivity to acid insoluble material was determined. Control - initiation mixture without puromycine. A. Absorbancy of suspension in medium CDGS. B. Absorbancy of suspension in germination medium.

time dependence of the puromycine effect of RNA synthesis. The result of such an experiment is illustrated in Fig. 4. Addition of puromycine to spores up to 6 minutes after initiation of germination decreases the rate of RNA synthesis by a factor of 5-6. On the other hand, puromycine has little or no effect if added 10 minutes following initiation of germination. The kinetic of the disappearance of the "puromycine effect" seems to be exponential. To correlate the apparent time of synthesis of the protein or proteins which affect RNA synthesis to germination, the decrease of absorbancy was measured during the experiment. Initiation of synthesis of this protein follows the decrease of absorbancy by 2-3 minutes and thus it may be one of the earliest proteins synthesized during differentiation of spore into bacterial cell.

The effect of puromycine on RNA synthesis is demonstrated both in CDGS-glucose medium which permits spore outgrowth to bacterial cells and germination medium which permits only germination and limited protein synthesis. Puromycine reduces rate of RNA synthesis in both media by a factor of 5-6. The synthesis of the protein which affects the rate of RNA synthesis is initiated at the same time in both media. The apparent slower disappearance of the effect of puromycine in germination medium may be explained by a lower rate of protein synthesis by spores incubated in this medium.

## DISCUSSION

The synthesis of a protein which partially controls transcription in outgrowing B. cereus spores is suggested by the results presented in this communication. The time of its synthesis - 2-3 minutes following germination, suggests that this protein may play a decisive role in the control of RNA synthesis, and through it - spore outgrowth to vegetative cell.

The nature of this protein is not yet demonstrated and it is possible that we are observing synthesis of additional RNA polymerase. However, preliminary experiments (A. Cohen, unpublished results) indicate that this protein controls not only the rate of RNA synthesis but also the nature of the species of the RNA synthesized. Our results suggest a system of positive control in spore outgrowth, similar to the one suggested by Losick and Sonenshein for cell sporulation.[2] Thus a process is conceivable in which the transcription activity of RNA polymerase is controlled both in sporulation and germination by a protein which is synthesized de novo early in these stages. This possibility is presently under investigation.

## REFERENCES

1. Steinberg, W. and H.O. Halvorson. J. Bacteriol. 95, 469-478, 1968.
2. Losick, R. and A.L. Sonenshein. Nature, 224, 35-37, 1969.
3. Nakata, H.M. J. Bacteriol. 88, 1522-1524, 1964.
4. Torriani, A. and C. Levinthal. J. Bacteriol. 94, 176-183, 1967.