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RELATION BETWEEN INHIBITION OF BACILLI SPORULATION AND SYNTHESIS OF LYTIC ENZYMES

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In two strains of *Bacillus*, the synthesis of two specific lytic enzymes was studied concomitantly with an inhibition of the sporulation : LD-carboxypeptidase synthesis was unaffected whereas γ-D-glutamyl-(L)*meso*-diaminopimelyl endopeptidase synthesis was shown to be closely related to sporulation. The endopeptidase production is totaly inhibited when netropsin inhibits sporulation in *B. sphaericus* and is low in *B. subtilis* Thy⁻A when sporulation is inhibited by thymidine starvation. This enzyme seems directly connected with the sporulation sequence.

INTRODUCTION

Early biochemical events during sporulation are initiated by various mecanisms, including the derepression of catabolite-sensitive promotor genes (1). The initiation of sporulation also coincides with the preferential synthesis of several enzymes (2, 3). In an earlier work, synthesis of a new endopeptidase active against the γ-D-Glu-(L)*ms*-A_2pm bond of the cortex tetrapeptide was shown in sporulating *Bacillus sphaericus* (4) and *Bacillus subtilis* (5). It was suggested that this enzyme plays a role in the synthesis of spore cortex (6). However it is difficult to determine whether the enzymic activity is really essential for sporulation. In this study we have used two methods for assessing the relevance of the γ-D-Glu-*ms*-A_2pm endopeptidase to sporulation. First we used an antibiotic, netropsin, which is known to be an inhibitor of sporulation (7, 8). On the other hand a strain of *B. subtilis* which only sporulates with addition of thymidine (9, 10, 11) was tested. In both cases the endopeptidase activity was analyzed. In the same way the activity of the L-lysyl-D-alanine (LD) carboxypeptidase present at all stages of vegetative growth and sporulation of *B. sphaericus* (4, 6) was studied.

MATERIALS AND METHODS

Organisms. *B. sphaericus* 9602, the medium and the conditions for obtaining maximum sporulation were described previously (4). *B. subtilis* Thy⁻A is a thymidine auxotroph kindly provided by Dr. Mandelstam (Oxford, England). This organism grows and sporulates with 20 µg of thymidine per ml of culture medium. It was grown with and without thymidine and harvested as described by

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Mandelstam *et al* (9) in the medium of Sterlini and Mandelstam (12) modified by Piggot (13).

Netropsin treatment. *B. sphaericus* was grown until the mid-log phase and netropsin was added at final concentrations ranging from 0 to 3 µg/ml. Microscopically distinct stages of sporulation were determined with a phase-contrast microscope and culture samples were collected.

Particulate enzyme preparations. They were obtained as described previously (5).

Radioactive substrates. The specific activity of N-acetyl muramyl-L-alanyl-γ-D-glutamyl-(L)*meso*-diaminopimelyl(L)-D-(^{14}C) alanine (substrate I) and of L-alanyl-γ-D-glutamyl-(L)*meso*-diaminopimelyl (L)-D-(^{14}C) alanine (substrate II) was 1.1 x 10^6 counts min^{-1} μmol^{-1}. The specific activity of N-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-lysyl-D-(^{14}C) alanine (substrate III) was 1.6 x 10^6 counts min^{-1} μmol^{-1}. They were obtained as described previously (4).

Enzymatic assays. Assays contained in 10 µl : 0.4 mM substrate, 26 mM, pH 8 Tris-HCl buffer, 8 mM $MgCl_2$ and 5 to 10 µg of protein per nmol of substrate. Incubations were performed at 37°C for 1 h. The products of the reaction were analyzed by thin-layer chromatography on cellulose in 1-butanol-acetic acid-pyridine-water (30:6:20:24) with the substrates I and II or in ethylacetate-pyridine-acetic acid-water (25:25:5:15) with the substrate III. They were detected by autoradiography and estimated as described in (4). Enzyme activities were expressed as nanomoles of *ms*-A_2pm-D-(^{14}C) Ala (from substrate I or II) and D-(^{14}C) Ala (from substrate III) released per mg of protein per hour.

Protein content. It was determined by the method of Lowry *et al* (14).

Materials. Netropsin was kindly provided by E.L. Patterson, Lederle Laboratories (U.S.A.).

RESULTS

LD-carboxypeptidase and endopeptidase activities in *B. sphaericus* grown with netropsin.

The effect of various concentrations of netropsin was tested on growth and sporulation of *B. sphaericus*. At the middle of the exponential phase, netropsin was added to the medium at final concentrations of 1, 2.2, 2.5 and 2.8 µg/ml ; a standard was grown without netropsin. The turbidity of the cultures was measured at 600 nm, the proportion of terminally swollen cells and refractile prespores was estimated by phase-contrast microscopy. The growth rate of *B. sphaericus* was not affected until a concentration of 2.2 µg/ml of netropsin, increasing concentrations of the antibiotic reduced the rate of growth. Sporulation was more sensitive to netropsin : at the concentration of 1 µg/ml the prespore formation was delayed and at $T_{6.5}$* it was only 30 % instead of 70 %

* T_n is time (in hours) after the end of exponential phase.

in the control. With concentrations of inhibitor greater than 2 µg/ml, the netropsin treated cells lost the ability to form refractile bodies. They also lost the ability to divide and appeared as long filaments. After T_6, strings of refractile bodies were seen in some of those filaments.

Samples of cells grown in the presence (2.2 µg/ml) and absence of netropsin were harvested 1 h before the end of the exponential growth and every 90 min from $T_{0.5}$ to $T_{6.5}$. Each sample was used for the preparation of enzymes. Enzyme preparations of cells grown without netropsin were used as control, two specific lytic enzymes were previously observed in such preparations : 1) an LD-carboxypeptidase split the L-lysyl-D-alanine linkage of lysine-containing substrates such as substrate III with release of free D-alanine. 2) a γ-D-glutamyl-(L)*meso*-diaminopimelate endopeptidase hydrolyzed the γ-D-glutamyl-(L)*meso*-diaminopimelic acid linkage of *meso*-diaminopimelic acid containing substrates I or II with release of the dipeptide *ms*-A_2pm-D-Ala (4). Such enzymic activities were searched in netropsin treated cells, results are shown in Fig. 1. The activity of LD-carboxypeptidase in the netropsin treated cells was similar to that found in the control samples and was roughly constant during exponential growth and sporulation. On the contrary the netropsin treated cells lacked entirely endopeptidase activity.

LD-carboxypeptidase and endopeptidase activities in *B. subtilis* thy⁻A strain grown without thymidine.

The effect of thymidine deprivation on growth and sporulation of *B. subtilis* Thy⁻A was tested following the method of Mandelstam *et al* (9). A culture containing thymidine (20 µg/ml) in casein hydrolysate medium (O.D.=2.0) was centrifuged and the cells were transferred to sporulation medium at a concentration of 0.25 mg dry wt/ml. Immediately, half of the culture was supplemented with 20 µg/ml of thymidine whereas the remainder was left deprived of thymidine. At intervals, samples were assayed for turbidity at 600 nm and for morphology. Hourly periods after induction of sporulation are denoted T_1, T_2 ... The growth of the cells deprived of thymidine reached a plateau at T_3 whereas in the presence of thymidine the cells grew linearly and were 50 % to contain refractile spores at T_6. In the cells that were deprived of thymidine some refractile spores were seen at T_6 (about 8 %). Samples were taken at $T_{1.5}$, T_3, T_5 and $T_{6.5}$. The corresponding particulate enzyme preparations were tested as above, the results are shown in Fig. 2. In cells starved of thymidine, LD-carboxypeptidase production was not affected. In the culture supplemented with thymidine the production of endopeptidase was similar to that found in the wild type strain (5) i.e. the activity was relatively low at $T_{1.5}$ and increased constantly during sporulation. By contrast the culture

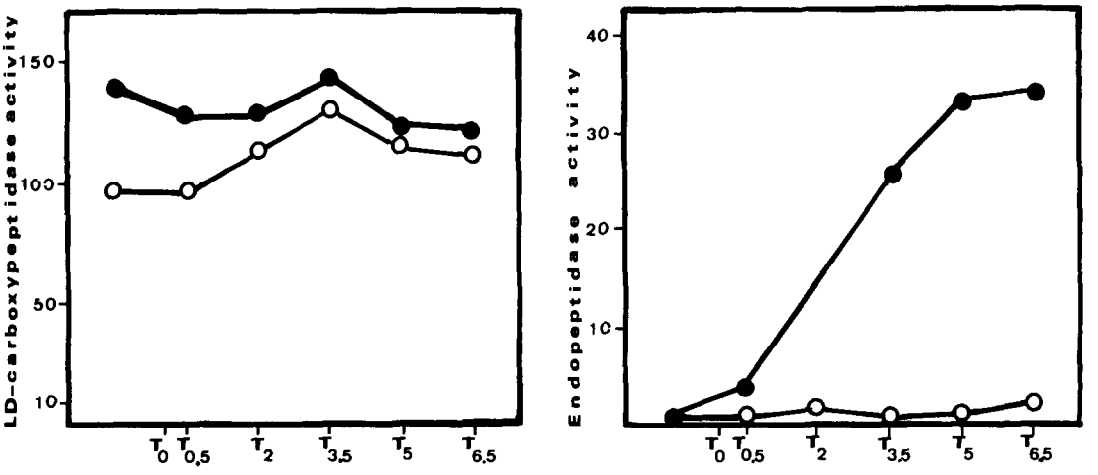

Figure 1. LD-carboxypeptidase and γ-D-Glu-*m*ś-A_2pm endopeptidase activities in *B. sphaericus* : ●—● without netropsin, ○—○ with netropsin (2,2 µg/ml). T_0 represents the end of the exponential phase and T_n are the hourly periods after T_0. LD-carboxypeptidase was assayed with the substrate III and endopeptidase with the substrate I. Enzyme activities are expressed as nanomoles of D-(^{14}C) Ala (LD-carboxypeptidase activity) or of *m*ś-A_2pm-D-(^{14}C)Ala (endopeptidase activity) released per mg of protein per hour.

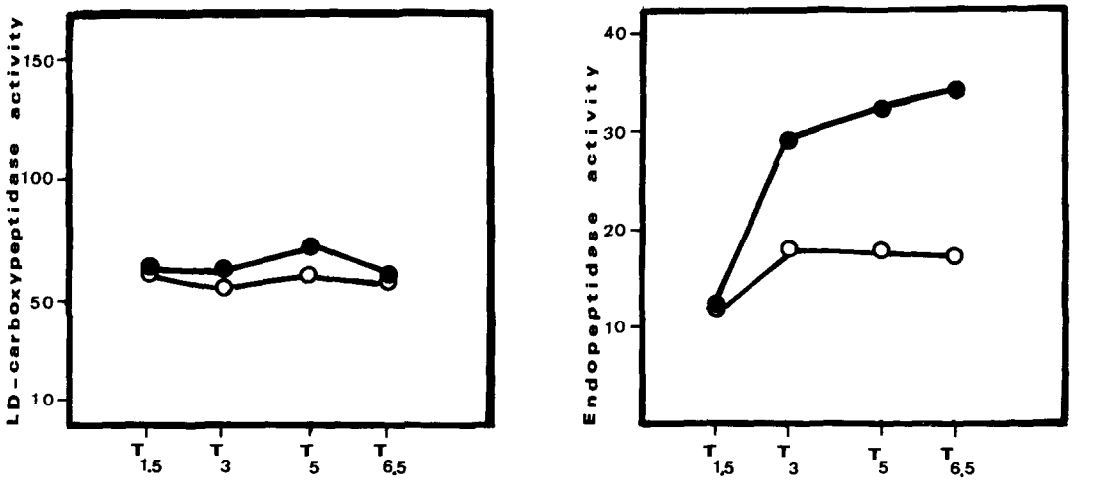

Figure 2. LD-carboxypeptidase and γ-D-Glu-*m*ś-A_2pm endopeptidase activities in *B. subtilis* Thy$^-$A : ●—● with thymidine (20 µg/ml), ○—○ without thymidine. T_0 represents the induction of sporulation. T_n are the hourly periods after T_0. LD-carboxypeptidase was assayed with the substrate III and endopeptidase with the substrate II. For the expression of the enzyme activities see legend for Fig. 1.

deprived of thymidine showed little increase of endopeptidase and this activity remained constant after T_3.

DISCUSSION

Studies have been done in view to determine whether certain functions that are non specifically derepressed during sporulation can be dissociated from specific sporulation processes.

(i) - Netropsin, a polypeptide antibiotic which inhibits sporulation but does not inhibit growth has been used by Keilman *et al* (7, 8) with *B. subtilis*. An analysis of the sporulation associated enzymes : proteases, aconitase, alkaline phosphatase and glucose dehydrogenase revealed that their rates of expression were not affected by the presence of the antibiotic. Only dipicolinic acid synthesis was prevented.

(ii) - A thymidine-requiring mutant derived from *B. subtilis* 168 (Thy^-A) has been used by Dancer and Mandelstam (11). In this strain, thymidine starvation, a condition known to inhibit sporulation (9) also inhibits the production of metalloprotease, serine protease, and ribonuclease ; α-amylase production, however, is unaffected. The authors suggested a classification of sporulation associated events in three categories : 1) events on the primary dependent sequence of sporulation, 2) events associated with sporulation but not essential for sporulation, 3) events unconnected with sporulation. Ribonuclease belongs either in category 1) or 2), metalloprotease is in category 2) and amylase appears to belong to the third category.

Our results show that the production of LD-carboxypeptidase is unaffected either in *B. sphaericus* when sporulation is inhibited by netropsin or in *B. subtilis* Thy^-A when sporulation is inhibited by thymidine starvation. Therefore LD-carboxypeptidase is unconnected with sporulation and belongs to the third category of enzymes suggested by Dancer and Mandelstam. This result was expected since LD-carboxypeptidase had been characterized previously during the log-phase of growth of *B. sphaericus* and throughout the sporulation process (4). On the contrary the γ-D-Glu-*ms*-A_2pm endopeptidase synthesis is totaly prevented in the presence of netropsin at a concentration that inhibits sporulation but not the growth of *B. sphaericus*. The result with the *B. subtilis* Thy^-A mutant deprived of thymidine is less probent. Nevertheless the production of the enzyme is two times lower than in control grown in presence of thymidine and it remains constant after T_3 whereas it increases in the control. This small production of enzyme can be related to an incomplete inhibition of the sporulation in the thymidine deprived culture (8 % of prespores). Thus the γ-D-Glu-*ms*-A_2pm endopeptidase seems tightly related to sporulation and should belong to the category 1 or 2 according to Dancer and Mandelstam (9).

This work confirms earlier results which had shown an important increase of the production of this enzyme during sporulation (4) and a delayed enzyme synthesis in a mutant with delayed sporulation (5). Our results are additional arguments for a role of the endopeptidase in the biosynthesis of the spore cortex.

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REFERENCES

1. Schaeffer, P., Millet, J., and Aubert, J.P. (1965) Proc. Nat. Acad. Sci. U.S.A. 54, 704-711.
2. Deutscher, M.P., and Kornberg, A. (1968) J. Biol. Chem. 243, 4653-4660.
3. Schaeffer, P. (1969) Bacteriol. Rev. 33, 48-71.
4. Guinand, M., Michel, G., and Tipper, D.J. (1974) J. Bacteriol. 120, 173-184.
5. Guinand, M., Michel, G., and Balassa, G. (1976) Biochem. Biophys. Res. Commun. 68, 1287-1293.
6. Tipper, D.J., Pratt, I., Guinand, M., Holt, S.C., and Linnett, P.E. (1977) Microbiology 50-68.
7. Keilman, G.R., Tanimoto, B., and Doi, R.H. (1975) Biochem. Biophys. Res. Commun. 67, 414-420.
8. Keilman, G.R., Burtis, K., Tanimoto, B., and Doi, R.H. (1976) J. Bacteriol. 128, 80-85.
9. Mandelstam, J., Sterlini, J.M., and Kay, D. (1971) Biochem. J. 125, 635-641.
10. Dancer, B.N., and Mandelstam, J. (1975) J. Bacteriol. 121, 406-410.
11. Dancer, B.N., and Mandelstam, J. (1975) J. Bacteriol. 121, 411-415.
12. Sterlini, J.M., and Mandelstam, J. (1969) Biochem. J. 113, 29-37.
13. Piggot, P.J., (1973) J. Bacteriol. 114, 1241-1253.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.