**Bacillus subtilis** Deoxyribonuclease Activity Specific for Single-Stranded Deoxyribonucleic Acid: Cellular Site and Variations During Germination and Sporulation

FABIO COBIANCHI, CARMEN ATTOLINI, ARTURO FALASCHI, AND GIOVANNI CIARROCCHI*

Laboratorio di Genetica Biochimica ed Evoluzionistica del Consiglio Nazionale delle Richerche and Istituto di Genetica, Università di Pavia, 14-27100 Pavia, Italy

The endonuclease of *Bacillus subtilis* specific for single-stranded deoxyribonucleic acid is absent in spores, appears during germination only after the start of deoxyribonucleic acid synthesis, and is located almost exclusively in the periplasm.

The main DNase of *Bacillus subtilis* that degrades single-stranded DNA can be fractionated into two forms which account for most of the total activity: a very high-molecular-weight endonuclease (greater than $5 \times 10^6$) and a low-molecular-weight endonuclease (36,000) (3). The higher-molecular-weight form can be converted almost quantitatively and irreversibly into the small enzyme by mild proteolytic treatment (4). A role for this sort of enzyme in physiological processes can be inferred from the observation that a recombination-deficient mutant of *B. subtilis* (rec30) (6) is reduced in this activity to approximately 13% of the wild-type level. The study of the cellular site of this enzyme is therefore relevant to its possible function in the handling of the incoming DNA, whether in transformation or in transduction. We have therefore studied whether protoplast formation releases the DNase in question into the medium, and we measured the level of this activity in the spore and at different times after sporulation.

Cells (1.3 g) of *B. subtilis* SB202 hisB2 tyrA1 trpC2 aroB2 were treated with lysozyme to destroy the cell wall and remove the soluble periplasmic fraction. In this way 80% of the total activity as measured by the acid solubilization assay was released (see Table 1). The remainder was extracted by subsequent osmotic shock and sonication of protoplasts. Scher and Dubnau also described a *B. subtilis* endonuclease activity localized in the protoplast supernatant, but this activity is specific for double-stranded DNA (7). The DNase specific for single-stranded DNA released by lysozyme (and therefore probably located in the periplasm) is fractionable into two molecular weight species, like the enzyme activity extracted by less specific treatment (whether alumina grinding or sonication) of whole cells. DNA polymerase I activity was monitored to show that internal enzymes are not released by the procedure used to obtain protoplasts of vegetative *B. subtilis* cells.

This observation raises the question whether the enzyme is present in the cell at a time when the cell wall is not yet formed, such as in the germinating spore.

We have therefore measured the level of this activity in the spore and at different times after sporulation (Fig. 1). The spor is practically devoid of single-stranded DNase activity (less than 1% of the log-phase value, expressed as units per genome). The level of the DNase begins to increase only after DNA synthesis has started, when the vegetative cell begins to emerge from the spore. The DNase level, corrected for DNA content, rises exponentially thereafter to reach a value of approximately 20

*Cells were grown in Bouillon Nutritif Complet (Bio Kar) and collected at an absorbancy at 560 nm of 1.5. Cells were washed with 80 ml of 0.05 M Tris-hydrochloride buffer (pH 7.5), 0.8 M sucrose, and 1 mM dithiothreitol, centrifuged at 4°C, and suspended in 10 ml of the same buffer. Lysozyme (40 mg) was added, and the suspension was incubated at 37°C. Protoplast formation was followed by phase-contrast microscopy. The suspension was centrifuged at 10,000 × g for 10 min, and the supernatant was stored at 4°C. Protoplasts were suspended in 3 ml of 0.01 M Tris-hydrochloride buffer (pH 7.5), 1 mM dithiothreitol, and 1 mM CaCl₂ and sonicated for 5 s at 0°C. The DNase activity was assayed using methods previously described (3). DNA polymerase I (Pol I) activity, defined as the *p*-chloromercuribenzoate-insensitive fraction of the total DNA polymerase activity, was assayed by the method of Ciarrocchi et al. (2). Protein was assayed by the procedure of Lowry et al. (5).
Fig. 1. Variations of the DNase activity during germination. Germination of strain PB202 was in the minimal medium of Spizizen (8) enriched with a combination of 0.2% Casamino Acids (Difco), 0.05% yeast extract (Difco), and 50 µg of tryptophan per ml (A) or in Bouillon Nutritif Complet medium (B). Samples were removed, and DNA content was determined as previously described (1). Crude extracts were prepared as follows: between 0.6 and 1 g of cells was sonicated for 30-s periods at 0°C in the presence of 4 g of glass beads, 4 ml of 0.05 M glycyl-glycine-NaOH buffer (pH 7.5), 10 mM dithiothreitol, and 2.4 mg of p-toluene sulfonfonyl fluoride. The suspension was then centrifuged (supernatant 1), and the precipitate was suspended in 4 ml of 0.01 M Tris-hydrochloride buffer (pH 7.5), 1 mM dithiothreitol, 1 mM CaCl₂, 0.5 M KCl, and 20% (vol/vol) glycerol, incubated at 37°C for 30 min in the presence of 8 mg of lysozyme, and centrifuged at 10,000 rpm for 10 min (supernatant 2). Total DNase activity, assayed as previously described (3), was the sum of the activities of the two supernatants. Spore preparation, germination, and harvesting of cells were as previously described (1).
to 30 units per $10^{10}$ genomes, which is maintained during logarithmic growth.

Spore breakage was confirmed by the release of DNA polymerase I activity (data not shown), as previously described (1). Thus the DNase activity seems specific for vegetative cells. During stationary phase, the overall DNase activity again rises by a factor of three to four, and then decreases when spores begin to form inside the cell (data not shown). It is not certain that this later increase is due to the same activity seen during log phase, since it is known that at this stage many exonucleolytic enzymes are excreted into the medium. In any case, the high level reached during stationary phase is in agreement with a possible role of the endonuclease in recombination, since cells show a high competence for transformation in stationary phase (9).

The defect in the rec-30 mutant is confirmed also if one looks only at the fraction of enzyme present in the periplasm; the specific activity of the single-strand specific DNase present in the protoplast supernatant is reduced to 13% of the wild-type value in this mutant, which is deficient only in recombination ability (transformation, transfection, and transduction) and not in repair properties.

The study of the molecular properties of the residual activity found in this mutant may shed light on its in vivo function.

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