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*A DEOXYRIBONUCLEASE FOUND AFTER INFECTION OF BACILLUS SUBTILIS WITH PHAGE SP3**

BY DAVID M. TRILLING† AND H. VASKEN APOSHIAN

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MEDICINE, TUFTS UNIVERSITY, BOSTON, MASSACHUSETTS

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SP3 is a bacteriophage capable of infecting transformable strains of *Bacillus subtilis*.[1, 2] The DNA of this phage contains thymine (unpublished observation). In order to understand the events which lead to the synthesis of mature phage, we have been investigating a number of viruses[3] which infect *B. subtilis*. After infection of *B. subtilis* SB19 with phage SP3, we have observed a 50-fold increase of a deoxyribonuclease (DNase) that degrades heat-denatured DNA to acid-soluble components. Evidence is presented that this increase is the result of the synthesis of a new protein after phage SP3 infection.

Materials and Methods—Materials: *B. subtilis* SB19 and phage SP3 were obtained from Dr. W. R. Romig. Calf thymus DNA, crystalline pancreatic ribonuclease, and crystalline pancreatic deoxyribonuclease were purchased from Worthington Biochemical Corp.; mitomycin C was purchased from Nutritional Biochemical Corp.; bovine plasma albumin from Armour Corp.; yeast sRNA type III was purchased from Sigma Chemical Co.; chloramphenicol was purchased from Parke, Davis and Co.

Methods: The medium used for growth of *B. subtilis* SB19 and for phage propagation was the TY broth described by Romig and Brodetsky[4] except that $CaCl_2$ was omitted and the $MnCl_2$ concentration was increased 10-fold. Lysates of phage SP3 were obtained by growing *B. subtilis* SB19 at 37° with vigorous aeration to a cell titer of approximately 1×10^8/ml. One phage per two cells was added and aeration was continued until the optical density of the culture decreased to approximately 10% of the value at the time of infection. The culture was then centrifuged at 14,000 × *g* for 10 min and the supernatant (approximately 1×10^{10} phages/ml) was saved. The phages were concentrated and partially purified by centrifuging 40-ml aliquots of the supernatant at 40,000 × *g* for 1 hr. The supernatants were discarded and each pellet was washed with 2 ml of phage diluant (0.01 *M* potassium phosphate buffer, pH 7.1; 0.01 *M* $MgSO_4$; 10 μg/ml bovine plasma albumin) and then resuspended by standing in 4 ml of phage diluant overnight at

4°C. The resuspended phages were passed through an HA (0.45 μ pore size) Millipore filter on which a layer of siliceous material (Hyflo Super Cel) had been placed. This sterile filtrate contained 1×10^{11} phages/ml.

To study the induction of the nuclease, *B. subtilis* SB19 was grown at 37° with vigorous aeration to a cell titer of about 1×10^8/ml. Crude lysates or partially purified phage preparations were utilized in induction experiments with identical results. Approximately 10 phages were added per cell. At appropriate times, 200 ml of the culture were quickly removed and poured onto crushed ice. The cells were collected by centrifugation, resuspended in 2 ml of cold buffer (0.05 *M* Tris-HCl, pH 7.5–0.002 *M* reduced glutathione), and disrupted by sonication with two 30-sec pulses using a Mullard ultrasonic generator. After centrifugation at 9600 $\times$ *g* for 30 min, the extracts were immediately assayed for enzyme activity. For enzyme purification 20 liters of infected culture were rapidly cooled 35 min after infection and immediately collected by centrifugation at 4°C in a continuous-flow DeLaval Gyro-Test centrifuge.

Induction of defective phages in *B. subtilis* SB19 by mitomycin C was performed as described by Seaman, Tarmy, and Marmur.[5] Extracts of mitomycin-treated cells for enzyme studies were prepared as above.

Isolation of DNA substrates: DNA was isolated from *B. subtilis* SB19 by the procedure described by Marmur,[6] with the following exceptions. Following the deproteinization of the lysed cell suspension, the aqueous phase was again deproteinized, centrifuged, and the nucleic acid was precipitated with ethanol. The nucleic acids were redissolved, digested with ribonuclease (RNase), and the procedure was continued as described.[6] The final product had a 260:230:280 optical density ratio of 1.00:0.47:0.54 and a molar extinction coefficient at 260 mμ of 6.94×10^3 based on deoxypentose.

To obtain P^{32}-labeled DNA from *B. subtilis* SB19, the cells were grown in 1 liter of a low phosphate peptone broth[7] containing 15 mc P^{32}. The labeled cells were harvested at an optical density of 1.50 at 600 mμ. The procedure for isolation of the DNA was based on the method of Lehman[8] through the RNase digest, after which the method of Marmur was used. The following modifications were employed: (1) lysozyme was used prior to sodium lauryl sulfate in lysing the cells; (2) the amount of RNase utilized was increased threefold; (3) 5 μmoles of unlabeled purified *B. subtilis* SB19 DNA was added after the RNase digestion; (4) after the chloroform deproteinization of the RNase digest, the aqueous phase was twice deproteinized, the final supernatant was clarified by centrifugation, and the DNA was precipitated with ethanol; (5) after the second isopropanol precipitation, the P^{32}-labeled DNA was dissolved in 8 ml 0.1 *M* NaCl, centrifuged at 80,800 $\times$ *g* in a Beckman model L-2 ultracentrifuge for 1 hr, and the supernatant was dialyzed overnight at 4°C against 6 liters 0.1 *M* NaCl. The isolated product is made acid-soluble by treatment with pancreatic DNase, but not by pancreatic RNase. The DNA had a molar extinction coefficient at 260 mμ of 6.99×10^3 based on deoxypentose and of 6.78×10^3 based on phosphorus.

Heat-denatured DNA was prepared by heating a solution of DNA (1 μmole per ml in 0.02 *M* Tris-HCl buffer, pH 7.5 and 0.02 *M* NaCl) at 100°C for 17 min and then immediately cooling it in an ice-water bath.

Concentrations of DNA are expressed as equivalents of nucleotide phosphorus unless otherwise indicated.

Deoxypentose was measured by the diphenylamine reaction of Dische,[9] and inorganic phosphate was determined by the method of Chen *et al.*[10] Protein was measured by the method of Lowry *et al.*[11] after precipitation with 10% trichloroacetic acid. All optical measurements were made with a Zeiss PMQII spectrophotometer. P^{32} was measured in a Nuclear-Chicago gas-flow counter with a Micromil window.

Assay of phage-induced DNase: The standard assay measures the conversion of acid-insoluble heat-denatured P^{32}-labeled *B. subtilis* DNA to acid-soluble fragments. The reaction mixture (0.30 ml) contains 20 μmoles Tris-HCl buffer, pH 8.0; 2 μmoles $MgCl_2$; 2 μmoles mercaptoethanol; 80 mμmole nucleotide equivalents of heat-denatured P^{32}-labeled DNA (3×10^5 cpm/μmole) and an amount of enzyme that liberates 5–30 mμmoles of acid-soluble product n 10 min. After incubation for 10 min at 37°C, the reaction mixture was chilled and 0.2 ml calf thymus DNA (2.5 mg/ml) and 0.50 ml 7% cold perchloric acid were added. After standing at 0°C for 8 min with intermittent mixing, the mixture was centrifuged for 5 min at 9600 $\times$ *g*. A 0.50-ml aliquot

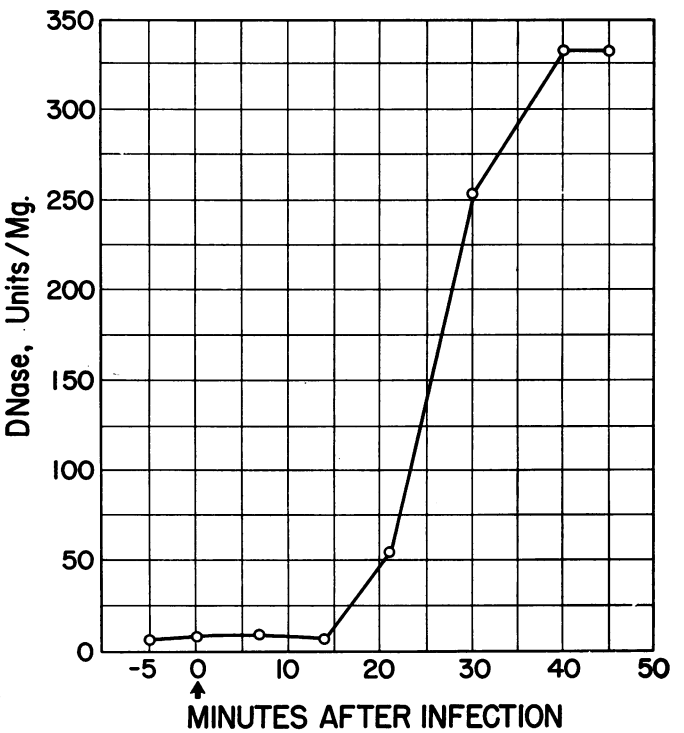

FIG. 1.—The increase in DNase activity after infection of *B. subtilis* SB19 with phage SP3. The arrow indicates the time of phage addition. Approximately 10 SP3 phages per cell were added to a culture of 1×10^8 cells/ml. Enzyme extracts were prepared and assayed as described under *Methods*. The OD_{600} of the culture was 1.94 forty min after infection and 0.60 sixty min after infection.

of the supernatant was placed on a planchet, neutralized with 3 drops of 1 *N* KOH, dried, and its radioactivity measured. Supernatant fluids from incubation mixtures without enzyme contained 0.60–0.75% of the added radioactivity. The radioactivity made acid-soluble was proportional to the amount of enzyme added; thus 0.005, 0.01, and 0.02 ml of an extract (diluted 1:5) liberated 716, 1430, and 2784 cpm, respectively, in the acid-soluble fraction. An enzyme unit is defined as 10 mμmoles acid-soluble phosphorus released in 30 min at 37°C. Specific activity is expressed as enzyme units per mg of protein.

The ultraviolet assay for DNase activity was performed in an identical manner except that the supernatant was transferred to a 1-cm quartz cuvette and the optical density at 260 mμ measured. An enzyme unit in this case was defined as 0.10 optical density unit of acid-soluble material released in 30 min at 37°C.

Results and Discussion.—Deoxyribonuclease activity of B. subtilis infected with phage SP3: After infection of *B. subtilis* SB19 with phage SP3, there is approximately a 50-fold increase in an activity that releases acid-soluble material from heat-denatured DNA (Fig. 1). The increase in activity over the low level found in uninfected cells is not detected at 14 min postinfection and reaches a maximum by 40 min postinfection when 340 enzyme units per mg protein are present. The time of appearance of this activity is later than that of the phage-induced dTMPase, an enzyme that can be detected 7 min after infection.[3, 12]

Similar results are obtained using assays based either on the formation of acid-

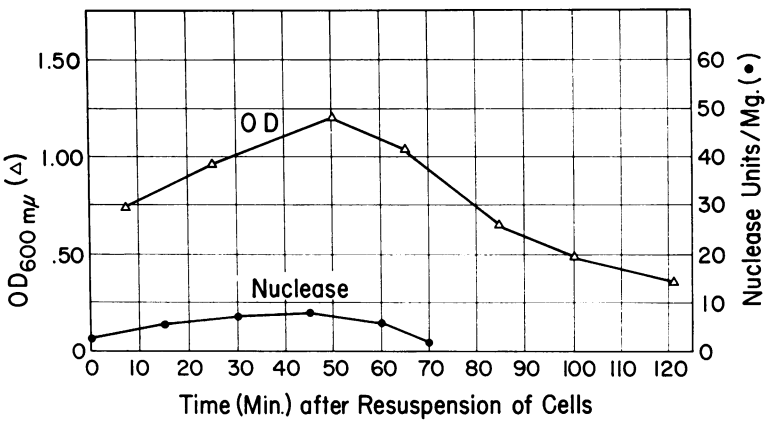

FIG. 2.—Induction of lysis of *B. subtilis* SB19 by mitomycin C and its influence on DNase activity. Following a 10-min exposure of logarithmically growing cells (OD_{600} = 0.50) to mitomycin C (3 μg/ml), cells were harvested, resuspended in the same volume of warmed medium, and reincubated. For enzyme assays, aliquots of the culture were poured on ice, harvested, and disrupted as described in *Methods*. The decrease in absorbancy is an indication of bacterial lysis.

soluble H^3 from *B. subtilis* SB19 DNA containing H^3-thymine or on the appearance of acid-soluble, ultraviolet-absorbing material.

The DNase, in the crude extracts of infected cells, attacks heat-denatured *B. subtilis* DNA at a rate 22 times greater than native *B. subtilis* DNA. When heat-denatured and native DNA's were used as substrates under identical conditions, 8.18 and 0.37 mμmole nucleotide equivalents were solubilized, respectively.

Chloramphenicol-inhibition of the appearance of phage deoxyribonuclease: When chloramphenicol is added 5 min after phage infection, no significant increase in DNase is observed (Table 1). The presence of chloramphenicol in the assay mixture does not inhibit the DNase activity. Hence, protein synthesis after phage infection is necessary for the appearance of the new DNase activity.

Failure of mitomycin C to induce phage deoxyribonuclease: Seaman *et al.*[5] have shown that treatment of *B. subtilis* with mitomycin C induces several morphologically distinct defective phages called PBSX and causes lysis. SP3 infection also induces defective *B. subtilis* phages.[13] In order to be certain that the DNase activity observed is not a consequence of SP3 inducing a PBSX-type defective phage (or of the induction of a temperate phage as, for example, the induction of λ exonuclease by mitomycin[14]), *B. subtilis* SB19 was treated with mitomycin C and the nuclease activity on heated DNA determined at various times. Treatment with mitomycin C resulted in lysis without any large increase in DNase activity (Fig. 2). The slight increase in activity (approximately threefold as opposed to 50-fold increase with SP3 infection) may be due to a change in host nuclease level with a change in the growth rate. The maximal level of nuclease activity observed in mitomycin-treated cells (7.3 units/mg) was similar to the level (7.5 units/mg) normally observed in untreated cells at the same titer of 1×10^8/ml. The presence of mitomycin C in an assay mixture had no effect on the SP3 phage-induced DNase.

The failure of pancreatic RNase to affect phage deoxyribonuclease assay and the constancy of RNase activity after infection: An increase in DNase activity after phage infection might be due either to the synthesis of a new DNase or also to the destruc-

TABLE 1

INHIBITION OF THE SYNTHESIS OF PHAGE SP3 DNASE BY CHLORAMPHENICOL

Cell extract	DNase activity (units/mg)
Uninfected	3.3
Infected	141.0*
Infected, chloramphenicol added 5 min after infection	4.0

From a culture at a titer of 1×10^8 cells/ml (OD_{600} = 1.50), three aliquots were removed. One aliquot was immediately chilled and the cells were harvested (uninfected cells). The other two aliquots received 5 phages per cell. Chloramphenicol (100 μg/ml culture) was added to one of these cultures 5 min after infection. Incubation was continued for 35 min, the infected cells were harvested, and extracts were prepared as described in *Methods*.

* Infection of *B. subtilis* SB19 at OD_{600} = 1.50 with an input multiplicity of 5 causes the production of one third as much enzyme as a multiplicity of 12. A multiplicity of 5 at OD_{600} = 2.0 causes production of an amount of enzyme similar to that produced at a multiplicity of 12 at OD_{600} = 1.50.

TABLE 2

FAILURE OF ADDED RNASE TO INFLUENCE DNASE ACTIVITY

Cell extract	DNase Activity (units/mg) Without pancreatic RNase	With pancreatic RNase*
Uninfected	7.0	8.1
35 min postinfection	394	390

* Added to the reaction mixture was 0.18 mg pancreatic RNase (heated for 5 min at 100°C to inactivate any contaminating DNase).

tion of some inhibitor of a pre-existing host DNase. For example, endonuclease I of *Escherichia coli* is inhibited by RNA.[15] If a similar RNA-inhibited DNase activity existed in *B. subtilis*, the synthesis of a phage-induced RNase might result in an increase in DNase activity. To determine if the DNase activity occurring after infection is sensitive to the presence of RNA, pancreatic RNase was added to reaction mixtures containing either uninfected or infected cell extracts. RNase did not affect the DNase activity in extracts of uninfected cells or in extracts of infected cells collected 35 min after infection (Table 2). Extracts of infected cells collected 20 min after infection also showed no effect of RNase on the DNase activity. Thus, the possibility of the progressive destruction of an RNA inhibitor appears unlikely.

Confirmation that the activity being studied is not the indirect result of an induced RNase is the absence of an increase in RNase activity after infection with SP3. When RNase activity was assayed with yeast sRNA as the substrate under the same conditions used for the ultraviolet assay of DNase activity, uninfected and 35-min postinfection extracts had 131 and 97 units of RNase, respectively.

Mixing experiments: Mixing experiments gave no indication of the presence of enzyme inhibitors in uninfected cell extracts nor of stimulators in infected cell extracts. Neither inhibition nor stimulation was detected after mixing uninfected and infected cell extracts over a three-fold concentration range (Table 3).

DEAE chromatography: SP3-infected cells, harvested 35 min after infection, or uninfected cells were disrupted and the nucleic acid was removed as described by Okazaki and Kornberg.[16] The preparations were dialyzed against 0.06 *M* K_2HPO_4–0.002 *M* reduced glutathione. Equal amounts of the dialyzed preparations from

TABLE 3

MIXING EXPERIMENTS WITH UNINFECTED AND SP3-INFECTED CELL EXTRACTS

Experiment	Acid-soluble P^{32} Nucleotides Formed (mμmoles) Uninfected	SP3-infected	Uninfected + SP3-infected
I	6.8	33.2	40.7
II	14.6	33.2	41.2
III	23.2	33.2	56.2

Reaction mixtures were as in the standard assay system (see *Methods*). In expt. I the aliquot of uninfected cell extract contained 64 μg protein. In expts. II and III, two or three times greater amounts of uninfected cell protein, respectively, were used. When used, 10 μg of protein from an SP3-infected cell extract were added to the reaction mixture.

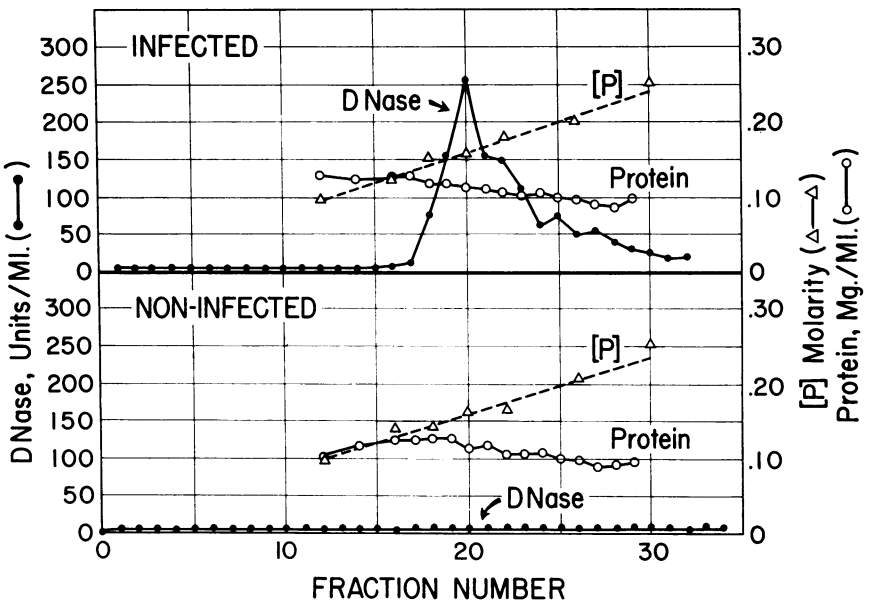

FIG. 3.—DEAE chromatography of the dialysates prepared from infected and uninfected cells after disruption and removal of nucleic acids. The size of each column was 1 × 15 cm. Columns were equilibrated with 0.06 *M* K_2HPO_4–0.002 *M* reduced glutathione, 20 mg of the appropriate fraction was added, and a linear gradient of 0.06 *M* to 0.35 *M* K_2HPO_4 was used for elution. Each reservoir (75 ml) also contained 0.002 *M* reduced glutathione. Fractions of 4.5 ml were collected. The recovery of SP3 phage-induced DNase was 67%.

infected and uninfected cell extracts were applied to two separate diethylaminoethyl-cellulose (DEAE) columns. A linear gradient of K_2HPO_4 was used to elute both columns. A distinct peak of DNase activity was present after DEAE chromatography of the SP3-infected cell fraction (Fig. 3). No such DNase activity peak was present in the preparation from the uninfected cells.

The peak of SP3 DNase activity after DEAE chromatography was found in fraction 20, which had a phosphate concentration of 0.16 *M*. Fraction 20 in the chromatogram of the uninfected cell fraction had an identical phosphate concentration, but no nuclease activity was detected in it or in any other tubes. When equal aliquots of the two fraction 20's were mixed, there was neither stimulation nor inhibition of the SP3 DNase.

Failure of $CaCl_2$ to replace $MgCl_2$: In contrast to a *Bacillus subtilis* SB19 DNase[16] which in cell extracts shows a 130-fold stimulation of activity when $MgCl_2$ is replaced by $CaCl_2$ (0.2 μmole per 0.3 ml of assay mixture), $CaCl_2$ between 0.025 and 0.50 μmole will not replace $MgCl_2$ in assaying the DNase in infected cell extracts. Similarly $CaCl_2$ at these levels could not be substituted for $MgCl_2$ in assaying the partially purified SP3 DNase from the DEAE column.

Increases in deoxyribonuclease activity have been described for *Escherichia coli* infected by T coliphages.[17–19] The induction of λ phage by treatment of K12 (λ) with mitomycin or ultraviolet radiation induces a λ exonuclease.[14] Crude extracts of SP10-infected *B. subtilis* have been reported to degrade DNA into acid-soluble material.[20] The role of these nucleases in the cell is not known. In the case of λ exonuclease, however, the synthesis of this enzyme appears to precede and be neces-

sary for vegetative replication of the λ genome.[21] A role for nucleases in λ infection has been proposed by Strack and Kaiser.[22] The role of SP3 phage-induced DNase is still unknown. The enzyme has been purified approximately 30-fold and is being further purified in order to study its properties, specificity, and function in the phage-infected cell.

Summary.—After infection of *B. subtilis* SB19 with phage SP3, there is an approximately 50-fold increase in DNase activity. This increase is prevented by chloramphenicol. Mitomycin C, an inducer of the defective phages of *B. subtilis*, does not induce the nuclease. The increase in nuclease activity does not appear to be due to the destruction of an inhibitor or synthesis of a stimulator. The new enzyme, which prefers heat-denatured DNA as a substrate, has been separated by chromatography on DEAE cellulose.

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