A Heat-Labile Inhibitor of Deoxyribonucleic Acid Degradation in Bacillus subtilis[1]

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Extracts of Bacillus subtilis can be prepared which have relatively little degradative activity on the transforming ability of homologous DNA(Reiter and Strauss, 1965). However, when such extracts are heated at temperatures of 70°C or above for short periods and then added to DNA, the transforming activity of the DNA is lost. In this report we should like to demonstrate that the phenomenon is due to the lability of a nuclease inhibitor.

Materials and Methods

Strain 168 of B. subtilis and its derivatives: uvr⁻ (Reiter and Strauss, 1965), mms⁻ (Searashi and Strauss, 1965), and MC-1(Okubo and Romig, 1966) were used in this study. All of these strains carry the ind⁻ marker. The prototrophic strain, B. subtilis W23, was also used. Extracts were prepared from cultures of the strains grown in CHT50 medium(Reiter and Strauss, 1965). The cells were harvested, washed with 0.5% NaCl + 0.5% KCl, suspended in 0.05M potassium phosphate buffer pH 7.4 to give a 2 to 3 per cent suspension (w/v) and lysed by treatment with 0.25 mg/ml of lysozyme. The extracts were treated with a Branson sonic disintegrator(one min., setting 7) to reduce viscosity and were then clarified by

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centrifugation for 30 min at 27,000 x g. Extracts were stored frozen at -60°C.

Nuclease activity was measured by the loss of transforming activity(Reiter and Strauss, 1965). Transformation assay for the indole locus was performed as described by Anagnostopoulos and Spizizen(1961) using B. subtilis DNA prepared by a modification of the method of Saito and Miura (1963). Competent cells, frozen and stored in 15% glycerol and which gave at least 0.5% transformation for the indole locus at saturating DNA concentration, were used. Protein was determined by the method of Lowry et al. (1951).

The reaction mixtures contained 8µg ind^+ DNA, 0.1 ml of extract, 0.1 ml of 0.01M $MgCl_2$, additions as indicated below and 0.05M potassium phosphate buffer, pH 7.5 to make 0.5 ml. Incubation was for 15 min at 37°C and was terminated by the addition of 0.1 ml of 2.5% sodium lauryl sulfate. The mixtures were kept cold until they were diluted 10^{-2} for transformation assay. At this dilution the detergent does not inhibit transformation and the number of transformants is a linear function of the DNA concentration.

Results

Transforming activity was lost when an extract from exponential strain 168 B. subtilis cells, heated for 10 min at 80°C, was added to ind^+ DNA. Addition of an equal amount of unheated extract to the reaction mixture inhibited the activity (Table 1). Ribonuclease treatment of unheated extract did not affect its ability to inhibit the heated preparation, but preliminary pronase treatment of the unheated preparation did destroy its ability to inhibit the nuclease. Pronase itself had no effect on the transformation assay since pronase added to DNA did not decrease the yield of transformants. Heat activation of activity has also been observed in preparations precipitated by the addition of ammonium sulfate to 80% saturation after removal of deoxyribonucleic acid by precipitation with streptomycin sulfate

Table 1. Heat activation of degrading activity

Heated extract	Other addition	Transformants/ml at a 10^{-2} dilution
0	0	4670 4150
+	0	20 30
0	unheated extract (3.0 mg protein/ml)	2200 1780
+	unheated extract	1630 1510
+	unheated extract stored at 37°C 1 hour	1980 1620
+	Pronase treated unheated extract	0 0
+	Ribonuclease treated unheated extract	2680 2450

Pronase treatment: 0.3 ml extract plus 0.3 ml heat treated (80°C - 10 min) pronase (1 mg/ml) for one hour at 37°C
Ribonuclease treatment: 0.3 ml extract plus 0.3 ml heat treated (80°C - 10 min) ribonuclease(1 mg/ml) for one hour at 37°C

Table 2. Effect of heat treatment

N_o extract	unheated extract	Extract heated 10 min at 50°C	60°C	70°C	80°C	90°C	100°C
3430	1460	2990	1330	0	2	3	1

recorded transformants per ml at a 10^{-2} dilution of the DNA

or with protamine. We therefore assume that the inhibitor is a protein.

Increased activity is observed after heating extracts at 70°C or above for 10 min (Table 2). Unheated extracts did show some degrading activity but this was

relatively minor compared to that of the heated preparations and was reduced by heating at 50°C. A precipitate forms on heating the extract but we have observed nuclease activity in the supernatant after centrifuging the heated extract.

Inhibitor is in excess in these preparations since 1:4 dilutions of unheated extract (3 mg protein/ml) inhibited the activity of undiluted, heated extract.

Table 3. Presence of inhibitor in extracts of different strains

Heated 168M exponential extract	Source of unheated extract	mg protein/ml in unheated extract	Transformants/ml at a 10^{-2} dilution
0	0	-	5710
+	0	-*	63
+	168(exponential)	3.0	3650
+	168(overnight)	22.	1870
+	MC-1(*rec*$_2^-$)	1.4	3140
+	59(*mms*$^-$)	2.9	2630
+	W23(overnight)	2.3	70
0	W23(overnight)	2.3	2820
+	*uvr*$^-$	1.7	1150

*The heated extract was prepared by heating the extract of strain 168 (exponential) for 10 min. at 80°C.

Inhibitor was found in extracts of a number of radiation sensitive strains(Table 3), including one, *mms*$^-$, which had been observed to degrade DNA at an excessive rate following irradiation(Strauss *et al.*, 1966). No inhibitor of nuclease activity was observed in an extract of an overnight culture of strain W23. Although W23 is an *ind*$^+$ strain, addition of the W23 extract did not appear to contribute additional

transformants, due perhaps to the degradation of DNA during the sonication step in extract preparation.

Discussion

We interpret the loss of transforming activity observed as due to the presence of a nucleolytic enzyme since loss of transforming activity has been shown to be an extremely sensitive method for the detection of single strand breaks(Lerman and Tolmach, 1959). Nuclease inhibitors have been reported in a number of systems (Laskowski, 1967) and a protein deoxyribonuclease inhibitor has been crystallized (Lindberg, 1966). The observation of an inhibitor in extracts of B. subtilis is interesting, however, since the protein inhibitors previously described have been observed in animal cells. The presence of such a nuclease and inhibitor in B. subtilis could be a factor in the integrity of DNA preparations prepared from this organism, particularly when a heat treatment is involved in the preparation(Marmur, 1961; Massie and Zimm, 1965).

References

Anagnostopoulos, C. and Spizizen, J., J. Bacteriol. 81, 744(1961)
Laskowski, M. Sr., Adv. Enzymol. 29, 165(1967)
Lerman, L. and Tolmach, L., Biochim. Biophys. Acta 33, 371(1959)
Lindberg, M., J. Biol. Chem. 241, 1246(1966)
Lowry, O., Rosebrough, N., Farr, A. and Randall, R., J. Biol. Chem. 193 265(1951)
Marmur, J., J. Mol. Biol. 3, 208(1961)
Massie, H. and Zimm, B., Proc. Natl. Acad. Sci. U. S. 54, 1636(1965)
Okubo, S. and Romig, W., J. Mol. Biol. 15, 440(1966)
Reiter, H. and Strauss, B., J. Mol. Biol. 14, 179(1965)
Saito, H. and Miura, K., Biochim. Biophys. Acta 72, 619(1963)
Searashi, T. and Strauss, B., Biochem. Biophys. Res. Commun. 20, 680(1965)
Strauss, B., Reiter, H. and Searashi, T., Rad. Res. Suppl. 6, 201(1966)