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Nature of the Ethylenediaminetetraacetic acid Requirement for Transformation of *Bacillus subtilis* with Single-Stranded Deoxyribonucleic Acid

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The ethylenediaminetetraacetate (EDTA) requirement for transformation of *Bacillus subtilis* with single-stranded deoxyribonucleic acid (DNA) was examined. The results indicate that a chelating agent such as EDTA is a stringent requirement for transformation with single DNA strands only at nonsaturating DNA concentrations, and that EDTA, when required, must be present during several steps in the transformation process and appears to insure the survival of single-stranded DNA by rendering a nuclease in competent populations inactive.

Denaturation of transforming deoxyribonucleic acid (DNA) produces two species of molecules: single DNA strands and reversibly denaturable native molecules (2, 6, 9, 12). Biological activity has been reported for both species (2, 6, 7, 9, 12). Extensive studies on the nature of reversibly denatured molecules present in preparations of denatured *Bacillus subtilis* DNA indicate that, under usual assay conditions, this molecular species is solely responsible for the transforming activity of denatured DNA (2, 6, 9, 12) and that single-stranded DNA is not taken up by competent cells (12). Preliminary studies by Chilton (7) indicated that single DNA strands exhibit biological activity on selected batches of competent cells in the presence of low concentrations of ethylenediaminetetraacetic acid (EDTA; 7).

The frequency of transformation with single strands of DNA is reported to be approximately 6% of the frequency obtained with an identical quantity of native DNA (7). Because of the low efficiency of transformation of single-stranded DNA as compared to helical DNA, it is essential for quantitative use of the single-strand transforming system either to identify conditions under which only the single DNA strands are biologically active or to remove reversibly denaturable molecules from preparations of denatured DNA.

In this report, we describe experiments which identify the EDTA-requiring steps in the transformation process and which indicate the mechanism by which the presence of EDTA promotes transformation with single-stranded DNA.

MATERIALS AND METHODS

Bacterial strains. *Bacillus subtilis* 168 (trp_2) was used as recipient in all transformation assays. A trp_2^+ transformant of 168 and the prototrophic strain W23 were used as sources of DNA.

Isolation and denaturation of DNA. DNA was isolated as described by Alberts (2). DNA samples were stored in 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.05 M EDTA (*p*H 8.8). By using this buffer, DNA can be denatured and partitioned in a dextran-polyethylene glycol two-phase system without prior dialysis. DNA was denatured after a four- to eightfold dilution in distilled water by addition of 0.1 volume of 1 M NaOH. After gentle mixing, DNA was allowed to stand for 5 min at room temperature before neutralizing with 0.1 volume of 1 M NaH_2PO_4.

Growth of competent cells and transformation. Competent cells were prepared by a modification of the two-step growth method (3). An overnight culture of *B. subtilis* 168 (trp_2) in Penassay Broth (Difco) was diluted fivefold into 40 ml of first-growth medium. The culture was incubated at 37 C with shaking (220 rev per min). The optical density of the culture was measured at 550 nm in a Bausch and Lomb Spectronic 20 colorimeter at 15-min intervals. Forty-five minutes beyond the cessation of logarithmic growth, the culture was diluted 10-fold into second-growth medium (transformation medium) supplemented with 50 μg of L-histidine and 50 μg of L-glutamic acid per ml. Both growth media contain 1×10^{-3} M $MgSO_4$.

Incubation was continued for 90 min. Cells were collected by centrifugation, suspended in single-strength *B. subtilis* salts (3) plus 10% glycerol, and frozen in 2.5-ml volumes by using a dry ice-ethyl alcohol bath. Competent cells were stored in a dry-ice chest. The transformation frequency with competent cells prepared in this manner was usually 0.5% for the try_2 marker at saturating levels of DNA. The frequency for transformation by single-stranded DNA was routinely 0.05 to 0.1 of this value.

For transformation assays, the frozen cells were thawed by shaking for 3 min at 37 C and diluted five-fold into transformation medium containing DNA. After 40 min of incubation at 37 C with shaking, the cells were diluted to give approximately 600 colonies per plate and plated in duplicate in 0.75% soft agar containing 0.1% acid casein hydrolysate on minimal agar plates. Transformation medium in which single strands of DNA were assayed contained 10^{-3} M EDTA. Disposable glass test tubes were used in all transformations. Transformations were performed with 0.1 μg of DNA per ml at *p*H 7.0.

Separation of native and single-stranded DNA. Reversibly denatured DNA molecules were removed from a preparation of alkali-denatured DNA by partitioning in a polyethylene glycol-dextran two-phase system (1). The phase stock solution used contained 16.8% (w/w) Dextran 500 (lot no. 4024; Pharmacia, Uppsala, Sweden) and 9.2% (w/w) polyethylene glycol 6000 (Baker Chemical Co., N.J.) in distilled water. This stock was kept frozen between experiments to restrict growth of contaminating bacteria. Before use, the stock solution was thawed at 37 C and then stirred rapidly at 4 C for 30 min to insure adequate mixing of the phases. Alkali-denatured DNA was diluted 10-fold in distilled water to reduce the phosphate concentration to 0.01 M. DNA (1 ml) was added to 0.95 ml of phase stock in a disposable glass test tube (13 by 100 mm). The tube was covered with parafilm and inverted gently 40 times at room temperature. The phases were separated by horizontal centrifugation at top speed for 20 min in an International Micro Centrifuge at 4 C. The polyethylene glycol-rich phase which contains predominately native DNA was transferred to a separate tube. The interface region was discarded. The dextran-rich bottom phase containing single-stranded DNA was retained in the tube. The DNA was transferred with a Biopette and disposable Biopette tips (Schwartz Bio-Research, N.Y.). Disposable glass tubes were used to minimize the adsorption of single-stranded DNA to glassware. Partitioned DNA was diluted usually five-fold with distilled water containing several drops of chloroform. The tube was inverted 20 times and centrifuged for 10 min to remove precipitated phase components. The resulting supernatant fluid was sterile.

Preparation and staining of DNA agar plates. Agar plates for detection of deoxyribonuclease contained 150 μg of native or alkali-denatured calf thymus DNA and 50 μg of acridine orange per ml of 0.75% agar in 10^{-3} M $MgSO_4$. Plates were examined under a mineral light. Native DNA stained green and denatured DNA stained orange. Nuclease was detected as an area of loss of fluorescence surrounding the colonies.

Materials. Pancreatic deoxyribonuclease (EC 3.1.4.5) was obtained from Worthington Biochemical (Freehold, N. J.) as the electrophoretically purified grade. All chemicals used were reagent grade. Solutions were made with deionized water distilled from an all glass apparatus.

RESULTS

Separation of single-stranded and reversibly denaturable DNA. Denatured DNA was partitioned in a two-phase polyethylene glycol-dextran system to separate reversibly denaturable molecules from single-stranded DNA and to identify the molecular nature of the DNA species mediating transformation when EDTA is present in the transformation medium (TME) and when it is withheld (TM). In this two-polymer system, native and reversibly denaturable DNA partition in the polyethylene glycol-rich top phase. Single DNA strands concentrate in the dextran-rich bottom phase (1). Native DNA was partitioned similarly to determine the distribution of native DNA in the separated phases. The results of partitioning native and denatured DNA are shown in Table 1. Eighty per cent of the biological activity in a preparation of native DNA migrated into the top phase, and 20% remained in the bottom phase. When the DNA remaining in the bottom phase was subjected to a second phase separation, 86% of this DNA moved into the top phase. Thus, after two cycles of partitioning, only 4.2% of the original native DNA remained in the bottom phase. When denatured DNA was partitioned in the two-polymer system, most of the transforming activity assayed in TM appeared in the top phase, indicating its native-like structure. Sixteen per cent of the activity remained in the bottom phase. Based on the distribution observed for native DNA in the two phases, this activity is accounted for as native DNA which failed to migrate into the polyethylene glycol layer. The biological activity of denatured DNA assayed in the absence of EDTA, then, is caused solely by reversibly denatured molecules (*also see* 2, 4, 7, 9, 12). Most of the biological activity in the denatured DNA assayed in TME before phase separating appeared in the dextran phase on partitioning, indicating that transformations under these conditions were mediated by single strands of DNA. These single DNA strands were 0.13 as active as native DNA in transforming *B. subtilis*. When DNA in the bottom phase was recycled in the two-phase system, a large proportion of the remaining reversibly denatured DNA moved into the top phase. Much of the single-strand activity in the dextran layer, however, was lost. Since the frequency of transformation by single DNA

TABLE 1. *Separation of single- and double-stranded deoxyribonucleic acid (DNA)*

DNA	Phase	EDTA[a]	Transformation frequency[b]	Ratio of EDTA present to EDTA absent	Per cent of total native activity[c]	
					In this experiment	In repeat experiments
Native	None	Absent	1.82×10^5	0.39		
		Present	7.12×10^4			
	Top	Absent	1.63×10^5	0.31	80	86, 81
		Present	5.07×10^4			
	Bottom I	Absent	4.4×10^4	0.36	20	14, 19
		Present	1.57×10^3			
	Top II	Absent	4.65×10^4	0.29	86	
		Present	1.35×10^4			
	Bottom II	Absent	7.66×10^3	0.32	14	
		Present	2.47×10^3			
Denatured	None	Absent	9.20×10^3	3.0		
		Present	2.80×10^4			
	Top	Absent	1.18×10^4	0.75	84	87, 90, 81, 86, 84, 87, 87
		Present	8.74×10^3			
	Bottom I[d]	Absent	2.28×10^3	9.4	16	13, 10, 19, 14, 16, 13, 13
		Present	2.14×10^4			
	Top II	Absent	1.47×10^3	0.44	67	60, 73
		Present	6.4×10^2			
	Bottom II	Absent	7.4×10^2	2.0	34	40, 27
		Present	1.45×10^3			

[a] Ethylenediaminetetraacetic acid at final concentration of 1×10^{-3} M when present.

[b] Transformants per milliliter per 0.1 μg of DNA.

[c] Ratio of transformants in top phase to transformants in top + bottom phases; concentrations at 0.1 μg DNA per ml.

[d] Bottom phase I was diluted fivefold in distilled water. The phosphate concentration was adjusted to 0.01 M before a second phase separation (top II and bottom II) as described in Materials and Methods.

strands decreases with decreasing molecular weight of the DNA (7), this reduction of biological activity during a second phase separation probably results from damage from shearing during handling.

In all subsequent experiments, reversibly denaturable molecules were removed from denatured DNA preparations by partitioning in the two-phase system.

EDTA-requiring steps in the transformation process. The influence of EDTA on binding of single-stranded DNA to cells was determined as follows. Single-stranded DNA was added to competent cells in TM and in TME. At intervals during the transformation period, portions of the transformation mixture from each tube were diluted 20-fold into TM and into TME, and the incubation was continued. This dilution is sufficient to reduce the level of EDTA below the concentration required to stimulate transformation by single DNA strands. After a total incubation period of 40 min, the entire contents of each tube was plated for trp_2^+ transformants. A DNA preparation with a high level of reversibly denaturable molecules was used so that the contribution of this fraction could be easily followed. The results obtained are presented in Fig. 1. Three features of transformation with single DNA strands are evident. When transformation is initiated in the absence of EDTA, transfer of cells after as little as 5 min to medium containing EDTA does not promote single-strand transformation. Since the concentration of DNA after the 20-fold dilution in TM is so low as to

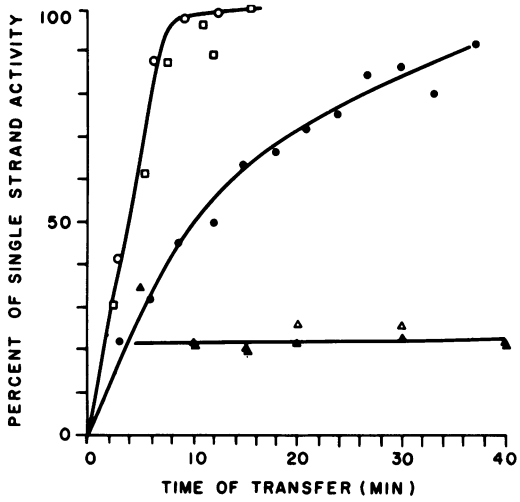

FIG. 1. *Effect of EDTA addition or withdrawal on transformation by single-stranded DNA. Per cent of single-strand activity is number of transformants per milliliter divided by the number of transformants per 0.01 ml when cells exposed to DNA in TME are shifted to TME at 12 min and then at the sampling times following. Transformation initiated in one medium and diluted at the time of transfer into second medium: begun in TM, dilute to TM (▲); begun in TM, dilute to TME (△); begun in TME, dilute to TM (●); begun in TME, dilute to TME (○ and □, separate trials).*

prevent a significant number of transformants arising from the binding of DNA at this period, it is concluded that EDTA is required either for binding of DNA to cells or for the protection of the DNA. Second, dilution of transformations begun in TME into the same medium reduces the number of transformants when the dilution occurs before 11 min. This finding suggests that either single-stranded DNA binds to cells slowly or that effective binding is reversed by dilution. Third, the frequency of transformation is reduced by removal of EDTA at any time before 32 min. Thus, EDTA is required during the period in which binding of single strands of DNA to the cell occurs and also enhances the frequency of transformation during later stages of the transformation process.

That the accessibility of the DNA bound to the cells to the action of deoxyribonuclease after the initial 12-min period is not changing in the absence of EDTA is demonstrated by the following experiment. Transformation was initiated in TME. After 12 min of incubation at 37 C to insure maximal binding of single DNA strands, the transformation mixture was diluted 20-fold into a series of tubes containing TM. At timed intervals, pancreatic deoxyribonuclease (10 μg/ml) and EDTA (1×10^{-3} M) were added to successive tubes. A similar dilution into TME without enzyme or $MgSO_4$ was included to determine the maximal single-strand activity. After a total incubation period of 40 min, the results were as shown in Fig. 2. Since the number of transformants remains unchanged with time after dilution into TM from the number obtained on dilution into deoxyribonuclease at 12 min, insensitivity does not continue to develop in the absence of EDTA.

To determine whether EDTA is required after development of deoxyribonuclease insensitivity to obtain a maximal transformation frequency with single-stranded DNA, a third medium shift was performed. In this experiment, transformation was initiated in TME. At 3-min intervals, portions were diluted 20-fold into TM and into TME, both containing deoxyribonuclease and $MgSO_4$. Transformants were assayed after a total incubation period of 40 min. In the event that EDTA was not required beyond the development of deoxyribonuclease insensitivity, the number of transformants surviving the exposure would be the same after dilution into TM or TME. The data are presented in Fig. 3. Enzymatic treatment followed by incubation in TM reduces the frequency of transformants over the value obtained on incubation in TME. In

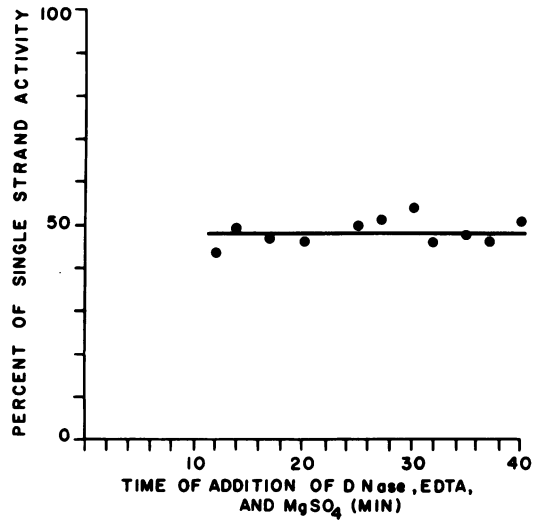

FIG. 2. *Effect of deoxyribonuclease on transformant frequency after 12 min of binding of single-stranded DNA in the presence of EDTA. Samples diluted from TME 20-fold to TM and 10 μg of deoxyribonuclease per ml, 1×10^{-3} M EDTA, and 3×10^{-3} M $MgSO_4$ (final concentration) added to samples at the indicated times. After 40 min of total incubation time, samples were plated for transformant count.*

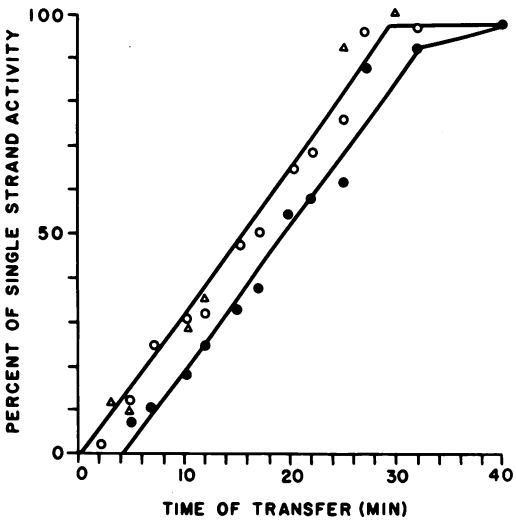

FIG. 3. *Requirement for EDTA for single-stranded DNA transformation beyond deoxyribonuclease insensitivity. Transformation initiated in TME, diluted at indicated time into TM (●) or TME (○, △, parallel experiments) containing 10 μg of deoxyribonuclease per ml and 3 × 10^{-3} M $MgSO_4$. Samples plated after 40 min of total incubation.*

addition, although 28 min of incubation in TME is sufficient to allow all assayable single DNA strands to attain insensitivity to deoxyribonuclease, 34 min of incubation in TM is required for the expression of transformation by single-stranded molecules to become independent of EDTA. The extent of the requirement for EDTA after the development of deoxyribonuclease insensitivity is emphasized by replotting the data as the per cent of the transformants which require EDTA after enzymatic digestion (Fig. 4).

Effect of calcium on the EDTA requirement. The inability to identify a particular step which requires EDTA in the single-strand transformation process suggests that EDTA may function simply by binding divalent cation. To test this possibility, portions of a transformation mixture begun in TME were diluted 20-fold into TME containing 10^{-3} M $CaCl_2$ at 3-min intervals. The result of shifting cells in the process of transformation into TME containing calcium (Fig. 5) is similar to that obtained on shifting into medium containing deoxyribonuclease. The coincidence of these data suggests that calcium is stimulating the activity of a nuclease present in competent populations of *B. subtilis*.

The dependence of reduction of single-strand transformation on the concentration of calcium in the medium is shown in Fig. 6. For this experiment, increasing amounts of $CaCl_2$ were added to TME and DNA before addition of competent cells. A transformation in TM and a transformation in TME containing 1 × 10^{-3} M $CaCl_2$ and an additional 1 × 10^{-3} M EDTA were also included. Concentrations of $CaCl_2$ above 5 ×

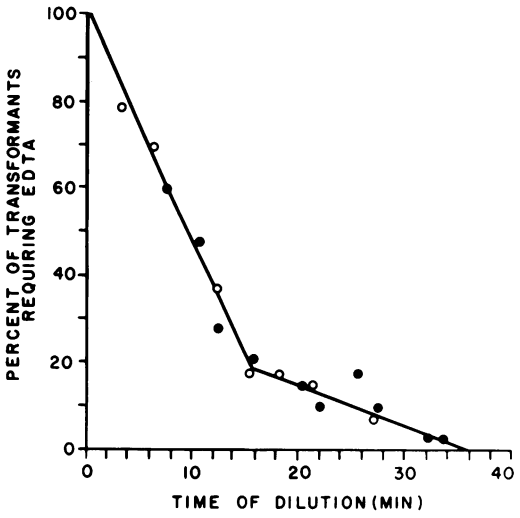

FIG. 4. *Percentage of deoxyribonuclease-insensitive transformants still requiring EDTA. Data of Fig. 3 plotted as transformants per milliliter assayed in TM containing deoxyribonuclease and Mg^{2+} divided by transformants/0.01 ml assayed in TME containing deoxyribonuclease and Mg^{2+} versus time of dilution from TME.*

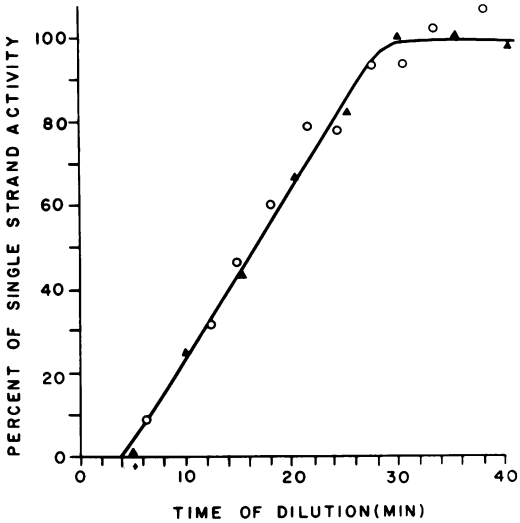

FIG. 5. *Effect of dilution of cells and single-stranded DNA from TME to TME containing 10^{-3} M $CaCl_2$ at different time intervals. Separate experiments, (○, ▲).*

10^{-4} M restrict single-strand transformation in the presence of EDTA at a concentration of 1×10^{-3} M. The observation that balancing the $CaCl_2$ added by adding an equal amount of EDTA restores transformation to the level obtained in TME indicates that the mechanism of action of EDTA involves binding of the divalent cation.

Effect of saturating DNA concentrations on the EDTA requirement. Figure 7 shows the response of competent cells to increasing concentrations of single-stranded DNA when EDTA is present and when it is absent from the transformation medium. In the absence of EDTA at concentrations of denatured DNA below 0.2 μg/ml, only the reversibly denaturable molecules mediate transformation. Above 0.2 μg of denatured DNA per ml, both native-like and some single-stranded DNA are assayable. At a concentration of 1.4 μg of denatured DNA per ml, the requirement for EDTA for maximal frequency of transformation with single-stranded DNA is abolished.

One explanation of the characteristics of the saturation curve and the response of single-strand transformation to calcium is that competent cells may produce a deoxyribonuclease with preference for denatured DNA which is inactivated either by binding single-stranded DNA or by removing divalent cations required for its activity.

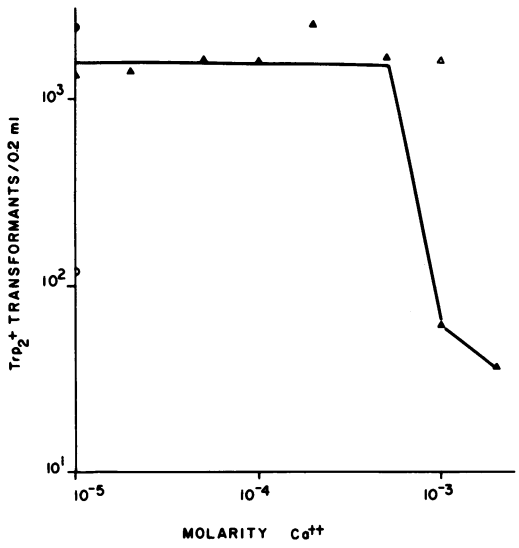

FIG. 6. *Effect of calcium concentration on transformation with single-stranded DNA. Cells were plated after 40 min of incubation in TM (○), TME (●), TM containing 1×10^{-3} M $CaCl_2$ and 2×10^{-3} M EDTA (△), TME containing the indicated concentration of $CaCl_2$ (▲).*

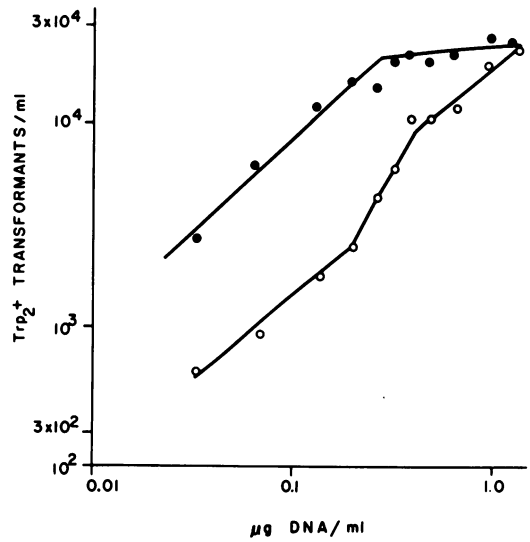

FIG. 7. *Effect of EDTA on transformation frequency as a function of increasing concentration of single-stranded DNA. Transformants after 40 min of incubation in TM (○) and in TME (●).*

Nuclease with preference for single-stranded DNA in competent populations. The existence of a nuclease with preference for single-stranded DNA was substantiated by spotting competent cells on DNA agar plates containing either native or denatured DNA. After 24 hr of incubation at 37 C, the plates were examined as described in Materials and Methods. Nuclease activity was detected as a clear zone around the area of growth on denatured-DNA agar plates. No clearing was observed around the area of growth of the same cells on native-DNA agar plates. Pancreatic deoxyribonuclease spotted on the same plates produced a zone of clearing on both native- and denatured-DNA agar plates.

That this nuclease is present both in the cell fraction and in the supernatant fluid of a competent population is shown by the data presented in Table 2. Cells grown to competence were pelleted by centrifugation. One-half of the cells were resuspended in fresh TM. The remainder were resuspended in the original culture supernatant fraction. Single-stranded DNA was added to each of these mixtures and trp_2^+ transformants were scored after 40 min of incubation. A third mixture containing single-stranded DNA and culture supernatant fluid was incubated for 15 min at 37 C before the addition of competent cells and EDTA. The results indicate that, in the absence of EDTA, either culture supernatant fractions or competent cells alone are capable of

TABLE 2. *Detection of single-stranded deoxyribonucleic acid (DNA)-specific nuclease in competent populations*

DNA[a]	EDTA added	Transformants per ml
	M	
Cells in fresh transformation medium	0	3.38×10^3
	1×10^{-3}	1.36×10^4
Cells in culture supernatant fraction	0	3.99×10^3
	1×10^{-3}	2.18×10^4
Culture supernatant fraction[b]	1×10^{-3}	2.24×10^3

[a] Phase-separated single-stranded DNA at 0.1 μg/ml.

[b] After 15 min at 37 C, competent cells and ethylenediaminetetraacetic acid (EDTA) were added. Incubation was continued for an additional 40 min before plating for transformants.

reducing the biological activity of single-stranded DNA.

DISCUSSION

The evidence presented in this paper indicates that the requirement for EDTA in promoting transformation of *B. subtilis* with single-stranded DNA is stringent at nonsaturating DNA concentrations and persists throughout binding, development of deoxyribonuclease insensitivity, and a later step(s) in the transformation process. These results differ from the observations obtained in the *H. influenzae* transformation system. In this latter system, EDTA is not required to detect transformation with single DNA strands but enhances transformation, presumably by aiding primarily in the step of penetration of single-stranded DNA into the cell (10, 11). In addition to the continued requirement for EDTA in the *B. subtilis* system, the observation that the EDTA requirement is abolished at concentrations of denatured DNA above 1.4 μg/ml reduces the feasibility of a similar "hole punching" step as the mechanism of action of EDTA (8) and, rather, suggests a requirement unrelated to stages of the transformation process.

The characteristics of the EDTA requirement can be explained by suggesting the presence of a single-strand specific nuclease in competent populations of *B. subtilis*. Such a nuclease has been reported in early stationary phase cultures of *B. subtilis* (4). This enzyme is characterized by a stringent requirement for divalent cation (4). Three lines of evidence indicate that the same or a similar nuclease is active in competent cultures. First, addition of calcium chloride to transformation medium containing EDTA abolishes single-strand transformation. Addition of excess EDTA to TME plus calcium ions permits transformation with single DNA strands at the maximal level, indicating that EDTA functions by removal of divalent cations which are detrimental to single-strand transformation. Second, high concentrations of denatured DNA promote transformations equally well in the presence and absence of EDTA. This result is expected when available nuclease is being saturated with single-stranded DNA. A similar protection of transforming DNA from the inactivating action of exogenous nuclease by helping DNA in the *Streptococcus* transformation system has been reported (5). Third, a nuclease with preference for denatured DNA is detectable in both the supernatant fraction of competent cultures and in competent cells resuspended in fresh medium.

EDTA, then, appears to function by removing from the medium divalent cations which are required for activity of nuclease produced and excreted by competent cells of *B. subtilis*.

ACKNOWLEDGMENTS

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