Competent *Bacillus subtilis* Cultures Synthesize a Denatured DNA Binding Activity

(Transformation/DNA uptake/nuclease)

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**ABSTRACT**  A protein activity has been detected in soluble extracts of competent cultures of *Bacillus subtilis* which protects denatured DNA from nuclease solubilization. Both binding activity and competence development occur simultaneously but not coordinate, since the peak of binding activity appears 3 hr after the peak of competence. The binding activity is not detected in physiologically noncompetent cultures, in an isogenic noncompetent mutant grown through the competence regimen, or in early sporulating cells. The activity is included on Sephadex G-100 gel filtration columns but elutes in a broad nonhomogeneous peak. This binding activity may play a role in uptake of DNA and/or integration of exogenous DNA into the genome of competent *B. subtilis* cells.

The fate of transforming DNA after exposure to competent cultures of two transformable bacterial species, *Bacillus subtilis* (1–4) and *Diplococcus pneumoniae* (5) has been studied in several laboratories. But the biochemical and physical properties of competent cells responsible for the events unique to transformation—the penetration of high-molecular-weight DNA through the cytoplasmic membrane and its integration into the recipient genome—have still to be identified.

Since we were interested in the problem of macromolecular transport through biological membranes, we turned to the *B. subtilis* transformation system to study the molecular mechanisms involved in DNA uptake by competent bacterial cells. In this report we present a preliminary characterization of a denatured DNA binding activity that is specifically synthesized in competent cultures and that may be part of the mechanism for transformation of competent *B. subtilis* cells.

**MATERIALS AND METHODS**

**Bacterial Strains and Competence Development.** A derivative of the *B. subtilis* 168 strain with the markers purA16, leu6, metB5, and ura, was used as the wild-type competent strain. An isogenic noncompetent strain was constructed by transformation of the wild-type strain with saturating levels of DNA prepared from the asporogenous and pleiotropic mutant *B. subtilis* 110NA (6). Ura3 transformants were selected and single colony isolates were screened for the asporogenous phenotype (smoothe colony) on Tryptose blood base agar (Difco). Asporogenous clones with the three remaining recipient cell markers were tested for lack of transformability and DNA uptake, and one of these clones was subsequently used.

**Abbreviations:** EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonylfluoride.

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Competence was developed by a two-step procedure (7). After growth of cells at 37°C in SI medium containing Spizizen salts (7), 0.02% casamino acids, 0.1% yeast extract, 0.5% glucose, and 20 µg/ml of required nutrients, cells were frozen in liquid N2 after addition of glycerol to 10% (v/v). These precompetent cells were quickly thawed at 45°C, centrifuged, and resuspended in twice the original volume of SII medium at 30°C. Except for the omission of yeast extract and leucine and the addition of 0.5 mM CaCl2, 2.5 mM MgCl2, and 0.05 mM spermine, SII is identical to SI.

**Extract Preparation.** Cells were washed by centrifugation in ice-cold buffer containing 20 mM Tris·HCl (pH 7.4), 10 mM MgCl2, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, and either 1 M or 50 mM KCl. Cells were routinely washed twice in the 1 M KCl buffer (to remove extracellular proteases) and then once in the 50 mM KCl buffer. Omission of the 1 M KCl buffer wash results in lower and less stable activity in cleared lysates. Washed cells were resuspended in a buffer containing 20 mM Tris·HCl (pH 7.4), 10 mM MgCl2, 5 mM EDTA, 5% glycerol, and 0.6 mg/ml of phenylmethylsulfonylfluoride (PMSF) and 5% ethanol (from the PMSF stock solution). The pH was adjusted upwards to 7.4 with a 10% (w/v) Tris solution after addition of PMSF. The resuspended cells (OD260 = 10–20) were sonicated with a Branson sonifier five times for 1 min in a rosette glass chamber immersed in an ice-water bath. The sonicate was centrifuged for 20 min at 104 rpm in a Sorvall RC-2B centrifuge, and this supernatant was centrifuged for 1 hr at 35,000 rpm in a Spinco 40 rotor. This high-speed supernatant is the cleared lysate and has a protein concentration of about 1 mg/ml. For the experiment in Fig. 2, a slightly different buffer, containing Triton X-100, and different sonication conditions were used, as detailed in the figure legend. As much as 2% Triton X-100 is without effect on binding activity.

[3H]DNA. Radioactive DNA was prepared according to Dubnau and Davidoff-Abelson (3) from *B. subtilis* 168 thy− trp− cells after growth in a medium of the following composition: Spizizen salts (7); 0.5% glucose; 0.5% casamino acids; 50 µg/ml of tryptophan; 2 µg/ml of thymine; 1 µM MnCl2; and 50 µCi/ml of [3H]thymine (23 Ci/mmol, Amersham-Buchler). The specific activity of the [3H]DNA was 4.5 × 106 cpm/µg of DNA. DNA concentration was estimated by a modified diphenylamine reaction (8). DNA (22 µg/ml in 0.015 M NaCl, 0.0015 M Na2·citrate, pH 7.0) was denatured by heating in a boiling-water bath for 10 min and then cooling in an ice-water bath. Disposable plastic tubes were used for all solutions containing denatured DNA. Trichloroacetic acid-insoluble radioactivity was determined by
filtering samples through 25-mm Whatman GF/C filters and washing five times with 5 ml of ice-cold 5% trichloroacetic acid and once with 15 ml of ice-cold 95% ethanol. Filters were dried and counted in 5 ml of toluene-based scintillation fluid [5.5 g of Scinti-Mix 4 (Koch-Light Laboratories Ltd.) per liter of toluene] on a Packard Tri-Carb model 2024 liquid scintillation counter. For determination of radioactivity in aqueous solutions, a 1:1 mixture of toluene-methanol with 5 g of 2,5-diphenyloxazole (PO) and 0.3 g of dimethyl-1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl PO-POPOP) per liter was used.

Nuclease Hydrolysis of DNA. Fifty millimolar Tris·HCl (pH 7.0) plus 10 mM MgCl₂ with 100 µg/ml of pancreatic DNase I and 10 µg/ml of snake venom phosphodiesterase (enzymes from Boehringer-Mannheim) was used to quantitatively solubilize aliquots of [³²P]DNA (usually 0.1 µg/ml). Samples were diluted into this nuclease mixture at 30°; after a desired time trichloroacetic acid was added to a final concentration of 5%, and the sample was cooled on ice, filtered, washed, and counted.

RESULTS

The Binding Assay. The assay for detecting an activity binding denatured DNA is based on the assumption that the DNA in a DNA-protein complex is less susceptible to nuclease hydrolysis than free DNA. This assumption is supported by reports that phage T4 gene 32 protein (φ), a single-stranded DNA binding protein, protects single-stranded DNA from hydrolysis by the exonuclease activity of both T4 DNA polymerase (10) and nuclease S1 (11). We have used a mixture of two commercially available nuclease, pancreatic DNase I (100 µg/ml) and snake venom phosphodiesterase (10 µg/ml), to detect proteins that protect denatured DNA from hydrolysis. This nuclease mixture solubilizes more than 99% of either native or denatured [³²P]DNA within 5 min at 30°. The kinetics of nuclease hydrolysis of denatured DNA in the presence of a soluble extract from competent B. subtilis cultures is seen in Fig. 1. A rapid initial rate of hydrolysis lasting for about 1 min is followed by a sudden leveling off until an asymptotic plateau has been reached between 5 and 10 min. By 15 min, 52% of the added DNA has been solubilized.

To demonstrate that the nuclease is still fully active when 48% of the DNA remains trichloroacetic acid-insoluble, more denatured DNA (but not more extract) was added at 11 min to a portion of the reaction mixture, and sampling of trichloroacetic acid-soluble material was continued. Fig. 1 shows that 91% of this DNA is solubilized by the nuclease within 15 min. Fig. 2 demonstrates that the amount of tri-
chloroacetic acid-insoluble material remaining after 15 min of nuclease hydrolysis is a linear function of the added extract concentration. Therefore, this measurement can be used to detect the presence of DNA-binding material both in crude extracts and throughout a purification of specific proteins. We have used the assay to detect changes in the levels of binding activity in cell extracts prepared from cultures developing competence.

There are some limitations to our assay. Since all cellular components with the ability to bind and protect denatured DNA from nuclease hydrolysis are simultaneously detected, it is not possible to assess the number of components involved. Likewise, quantitative and qualitative differences in binding of different components to DNA in a mixture cannot be determined.

The Competent Specific Nature of the Binding Activity Detected in Cleared Lysates. The DNA binding activity was measured as described above in cleared lysates prepared from cultures of B. subtilis developing competence. We found that as the transformativity of the culture increases, reaching its maximum level in 3–4 hr at 30°, the level of binding activity also increases (Fig. 3). Two slightly different competence-eliciting growth media were used in the experiment shown in Fig. 3. In one case the culture was supplemented with yeast extract to permit good outgrowth of cells after competence development and emergence from the lag phase. The other culture was not supplemented with yeast extract and never fully emerged from the lag phase. Competence, as measured by the transformation assay, is twice as high but less stable in the former culture than in the one not supplemented with yeast extract. Despite the 2-fold difference in levels of transformation between the two cultures, little difference is observed in the amount of binding activity in extracts of these cells. Both the rate of increase and final levels of binding activity appear to be very similar under both conditions. In fact, binding activity continues to increase for another 2 hr in both cultures even though the levels of transformation have begun to decrease. This lack of coordination between the appearance of binding activity and of transformativity may mean that the level of binding activity is not a unique determinant of the competent state. When the binding activities are expressed as μg of DNA bound per transformant at the peak of competence (4 hr), the values are 9 × 10−8 and 1.6 × 10−8 for the cultures with and without yeast extract, respectively. Cahn and Fox (12) have reported values ranging between 2 and 3 × 10−8 for DNA uptake by purified competent cells. Thus, lysates from competent cultures are capable of binding at least as much DNA as would normally be taken up by the competent cells.

When a pleiotropic asporogenous mutant of B. subtilis isogenic with the wild-type strain but unable to develop competence, is grown through the competence regimen, there is no increase in binding activity above the initial starting values (Fig. 3). The difference in binding activity between the mutant and wild-type cells at 4 hr is a factor of 18. At 3 hr the wild-type cells are more than 100 times more transformable than the mutant. Extracts from wild-type cells grown in broth medium (in which no competence develops) have no activity above the starting levels indicated in Fig. 3. Extracts prepared from early sporulating cells (at T1 and T2) also have no binding activity. Furthermore, wild-type log phase cells growing in a minimal salt medium similar to SII have the same low level of activity as noncompetent cells (data not shown). Since only the competence regimen elicits the synthesis of a high level of denatured DNA binding activity in wild-type cells and since the identically grown isogenic mutant never develops this activity, we conclude that most of this single-stranded DNA binding activity is specific to the competent state.

When extracts are prepared from competence-developing cultures over a 12-hr period, a peak of activity is detected around 6 hr which, thereafter, decreases (Fig. 4). Development of binding activity is sensitive to the antibiotics rifampicin (5 μg/ml) and chloramphenicol (100 μg/ml) at concentrations that normally inhibit transcription and translation, respectively. Thus, we conclude that the activity is synthesized de novo in competent cultures.

Ammonium Sulfate Fractionation. Cleared lysates from competent cultures were fractionated by ammonium sulfate in two steps: 0–40% and 40–60% saturated ammonium sulfate solutions. Although 80–90% of the measured binding activity precipitated in the second step with a specific activity increase of 2 to 3, not all of this activity proved to be competent-specific (see below). This ammonium sulfate fraction was used for further characterization.

Salt Sensitivity of the Binding Step. Since high salt concentrations inhibit nuclease activity, it proved difficult with our assay to determine directly the sensitivity of the DNA-binding activity to high ionic strength. When denatured DNA was incubated with the ammonium sulfate fraction at NaCl concentrations ranging from 50 mM to 1.5 M and then diluted 20-fold into the usual nuclease mixture, as much as 75% less DNA remained nuclease-resistant at the higher salt con-
Ammonium Sulfate coincident of aggregates of DNA recovery a emerges at fraction. Protein is the weight concentrations. The residual 25% activity may represent either a recovery of binding upon dilution into the nuclease (although the weight ratio of nuclease to ammonium sulfate fraction protein is 400 to 1) or a high ionic strength resistant binding of DNA by certain components of the ammonium sulfate fraction.

Molecular Weight Estimation and Heterogeneity of the Ammonium Sulfate Fraction. When the 40–60% ammonium sulfate fraction was applied to a Sephadex G-100 column, two peaks of activity emerged (Fig. 5, lower part). The first peak is coincident with the void volume as determined by a blue dextran marker, and the midpoint of the second, broad peak, emerges at about the same point as chymotrypsinogen A (molecular weight 45,000). The broadness of the second peak suggests either the presence of more than one component or aggregates of a single component. This broad, nonhomogeneous peak is, however, competence-specific, as shown by the following experiment. When an identically prepared ammonium sulfate fraction from an extract of log phase cells grown in broth medium is run on the column, the only measurable activity to emerge runs with the void volume (Fig. 5, upper part).

These gel filtration results reveal that only 50% of the measured activity in the ammonium sulfate fraction prepared from competent cell lysates is unique to the competent cells. It appears, then, that our ammonium sulfate fractionation of cleared lysates does not precipitate the competence related binding activity as efficiently as the background noncompetent activity. Most of the protein applied to the column elutes with the void volume (open circles in Fig. 5), and we calculate that the specific activity of the middle fractions in the second peak is 10 times higher than the specific activity of the fractions eluting with the void volume.

DNA–Cellulose Chromatography. In principle it should be possible to isolate a DNA binding activity in a highly purified state by affinity chromatography on DNA–cellulose columns as described by Alberts and Herrick (13). However, we were unable to elute this binding activity from a denatured DNA–cellulose column prepared and run according to Alberts and Herrick (13). DNA–agarose columns (14) and batch techniques using DNA–agarose were also tried without success. There are three possible explanations for the failure of the affinity chromatography technique: (i) Since DNA is not covalently bound to either agarose or cellulose, very small amounts of single-stranded DNA might be eluted from the column and might lead to negative results with our assay. (ii) The binding protein(s) in lysates of competent cultures may be irreversibly bound to the single-stranded DNA on the column. (iii) High salt concentrations may inactivate the protein(s) during elution from the column.

**DISCUSSION**

Only one strand of a donor DNA molecule is integrated into the recipient cell genome after DNA uptake (3, 15–17). How and where strand separation occurs during the process of uptake and integration in *B. subtilis* is presently not known. The single-stranded DNA that can be released from a complex in lysates of competent cells after DNA uptake has a molecular weight similar to the integrated piece of DNA. Thus, it seems possible that the complexed DNA is a precursor of the integrated DNA segment (2). The kinetics of appearance of single-stranded DNA after uptake supports this view (1, 2). Of interest to our study is the fact that Piechowska and Fox (1) found complexes of denatured DNA similar to those extracted from competent cells after DNA uptake by lysing...
cells in the presence of denatured DNA. The competent-specific nature of the complex was not demonstrated, nor was its DNase-resistant state examined. It seems possible that the Piechowska-Fox complex is similar to the DNase-resistant state of denatured DNA that we find after exposing denatured DNA to competent cell lysates.

An intracellular DNA-protein complex in competent cells can play at least two roles in the transformation process. If DNA penetrates the cell membrane as a single-stranded molecule, the association of DNA binding proteins with the incoming strand would favor continued uptake of the molecule (which is five times the length of the cell) and protect it from nuclease inactivation. The complexed DNA may also represent the physical state of the single-stranded DNA molecule just prior to integration. For example, recombination between DNA molecules in T4-infected Escherichia coli requires functional T4 gene 32 protein (9). Meiotic cells of Lilium synthesize a single-stranded DNA binding protein which is thought to be involved in recombination events unique to the meiotic state (18). A similar meiotic DNA binding protein has also been found in mammalian spermatocytes (19). Thus, DNA-protein complexes seem to be integral and ubiquitous parts of recombination mechanisms.

Further work on the single-stranded DNA binding activity found in competent B. subtilis cells is needed. The details of its physical and chemical nature, the regulation of its synthesis, and its biological role(s) should be characterized in order to understand better the coordination between uptake and integration of foreign genetic material in bacterial cells.

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