Effects of pH on Transformation of *Bacillus subtilis* with Single-Stranded Deoxyribonucleic Acid

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Variation in frequencies of transformation mediated by native and single-stranded DNA and its dependence on pH of the medium were investigated. The results indicate that the biological activity of deoxyribonucleic acid (DNA) of both configurations assayed in the presence of ethylenediaminetetraacetic acid (EDTA) increases as the pH of the transformation mixture is lowered from 7.7 to a maximum transformation frequency near pH 6.1. At this lower pH, native DNA transforms equally in medium with and without EDTA, and single-stranded DNA is 0.4 to 0.6 as active as native DNA in transforming *Bacillus subtilis*. A high efficiency of transformation with single-stranded DNA was observed for five markers in three recipient strains. The increased efficiency of native DNA appears to be caused by a lesser capacity of EDTA to bind magnesium at the lower pH. The increased efficiency of single-stranded DNA at pH below 7.0 results from decreased activity of a single-strand specific nuclease present in competent populations.

Competent *Bacillus subtilis* 168 cells are transformed to prototrophy with single-stranded deoxyribonucleic acid (DNA) when ethylenediaminetetraacetic acid (EDTA) is present (2, 3, 6). EDTA appears to enhance transformation with single-stranded DNA by removing divalent cations which stimulate activity of a single-strand specific nuclease produced and excreted by competent cells (6).

Single-stranded DNA is usually one-tenth as active as native DNA in transforming *B. subtilis* at limiting DNA concentration (6). The low efficiency and the confinement of this transformation of the *trpC2* marker in the 168 recipient strain (2, 3, 6) have limited the usefulness of this technique as an assay of single-stranded DNA.

In this report, we will show that selection of an appropriate pH increases the efficiency of transformation by single DNA strands relative to double-stranded DNA. The results indicate that the biological activity of single-stranded DNA increases as the pH of the transformation medium is lowered from pH 8.0. Single-stranded DNA is 50% as efficient as native DNA in transformation assays under the optimal conditions described below. In addition, we demonstrate efficient transformation of markers in additional recipient strains by single-stranded DNA. The effects of lowering the pH of the transformation medium on various parameters of the transformation assay are described.

MATERIALS AND METHODS

**Bacterial strains.** *B. subtilis* 168 (*trpC2*), BR151 (*trpC2*, *lys-3*, *metB10*), and Mu8u5u6 (*purB6*, *lev-8*, *metB5*) were used as recipients in transformation assays. The prototrophic strain W23 was the source of DNA.

**Isolation and denaturation of DNA.** DNA was isolated, stored, denatured, and separated from reversibly denatured molecules, using the procedures described previously (6). Denatured DNA from which reversibly denatured molecules were removed is referred to as single-stranded DNA to distinguish it from bulk denatured DNA.

**Growth of competent cells and transformation.** Cultures were grown to maximal competence and transformed as described previously (6), except that 10$^{-4}$ M CaCl$_2$ was included in transformation assays performed in the absence of EDTA. All transformation assays were performed using 0.1 µg of DNA per ml.

**RESULTS**

**Variation in transformation frequency with pH.** The frequency of transformants mediated by native and single-stranded DNA in transformation media (TM) ranging in pH from 8.0 to 4.5 was investigated. Competent cells which had been frozen and thawed in single-strength *B. subtilis*
salts (1) plus 10% glycerol were diluted fivefold into TM adjusted to various pH values and containing 0.1 μg of DNA per ml. After 40 min of incubation at 37 C, the number of trpC2+ transformants was assayed by plating on selective media. All transformations were performed simultaneously using the same DNA and competent cell preparations. The results obtained and the contributions of the cells in B. subtilis salts to the final pH of the transformation mixture are presented in Fig. 1 and 2. The frequency of transformants assayed in TM using native DNA was not affected by a variation in final pH between 5.8 and 7.7. Native DNA assayed in medium containing EDTA (TME) exhibits increasing biological activity as the pH of the transformation mixture is lowered from 7.7 to a maximal frequency near pH 6.1. The activity of native DNA assayed in TME is twofold higher at pH 6.1 than at pH 7.0. The biological activity of reversibly denatured DNA molecules remaining in the preparation of single-stranded DNA (single-stranded DNA assayed in TM) parallels the response of native DNA to variations in pH. The number of transformants obtained by using single-stranded DNA assayed in TME increases as the pH of the medium is lowered and attains a maximum value near pH 6.1. The efficiency of single-stranded DNA is approximately four times greater at pH 6.1 than at 7.0.

Efficiency of single-stranded DNA using various recipient strains. The efficiency of single-stranded DNA assayed in TME at pH 6.1 was compared to that of an equal quantity of native DNA assayed in TM on the 168, BR151, and Mu8u5u6 recipient strains. The single-stranded DNA was prepared with a minimum of handling and holding before adding to recipient cells to lessen damage caused by shear and losses of the single-stranded DNA adsorbing to the glassware. The results appear in Table 1. Each marker in the three strains is transformed by single-stranded DNA. The efficiency of this DNA is 40 to 60% that of native DNA with B. subtilis 168 and BR151 as recipients. Markers in the Mu8u5u6 strain are transformed by single-stranded DNA with a lower efficiency.

Effect of added magnesium on transformation with native and single-stranded DNA. Addition of EDTA to TM reduces the frequency of transformation with native DNA by removing magnesium (3) which is essential to the transformation process (7). Readdition of magnesium to TME restores the original biological activity of the DNA (3). A possible explanation for the increased activity of native and single-stranded DNA at pH 6.1 is the reduced capacity of EDTA for magnesium at the lower pH. This explanation presumes a similar magnesium requirement during transformation by DNA of both configurations. The requirement for magnesium during transformation with single-stranded DNA was investigated.
TABLE 1. Transformation of various strains of Bacillus subtilis with single-stranded DNA

<table>
<thead>
<tr>
<th>Strain</th>
<th>DNA</th>
<th>Transformants/ml*</th>
<th>Per cent of native**</th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td>Native</td>
<td>1.86 x 10^4</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Single stranded</td>
<td>7.65 x 10^4</td>
<td>58, 54, 20, 45, 48</td>
</tr>
<tr>
<td>BR151</td>
<td>Native</td>
<td>3.81 x 10^4</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Single stranded</td>
<td>2.44 x 10^5</td>
<td>51</td>
</tr>
<tr>
<td>Mu8u5u6</td>
<td>Native</td>
<td>2.91 x 10^4</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Single stranded</td>
<td>6.38 x 10^4</td>
<td></td>
</tr>
</tbody>
</table>

*Native DNA assayed in TM: single-stranded DNA assayed in TME. All assays were performed by using 0.1 μg of DNA/ml and at pH 6.1.

**Per cent of native = (transformants/ml with native DNA in TM)/(transformants/ml with single-stranded DNA in TME) x 100 for trpC2* in 168 and BR151: purB6* in Mu8u5u6.

by initiation transforming in TME at pH 7.0 without magnesium and diluting 20-fold into TME containing 10^-3 M MgSO4 at 5-min intervals. The diluted competent cells and the B. subtilis salts contribute 2 x 10^-4 M MgSO4 to the transformation assays performed in TME without added magnesium. After 40 min of incubation, 3 ml of soft agar was added to each tube, and the contents were poured onto the surface of minimal agar plates. An identical transformation assay was begun in TME containing magnesium and diluted into the same medium after 15 min of incubation [at which time the number of transformants obtained is unaffected by dilution (6)], and this regimen produced 2.02 x 10^4 trpC2 transformants/ml. The percentage of this total number of transformants obtained at each time of dilution into TME and magnesium is expressed in Fig. 3. These results indicate that magnesium is needed to obtain the maximum number of transformants after the first 10 min of incubation. The observation that magnesium can be omitted during the initial 10 min of the transformation process suggests that this cation is not essential during the period in which binding of single-stranded DNA occurs.

Table 2 presents the effect of additional magnesium on transformation with native and single-stranded DNA at pH 7.0 and 6.1 in a series of transformations performed in TM, TME, and TME containing an additional 10^-3 M MgSO4. The decrease in biological activity of native
DNA assayed in the presence of EDTA at pH 7.0 is prevented by adding excess magnesium. Neither the presence of EDTA nor additional magnesium alters the number of transformants produced by native DNA at pH 6.1, indicating that TME at the lower pH contains sufficient magnesium available for maximal transformation with native DNA. Addition of magnesium to TME does not increase the frequency of transformation with single-stranded DNA at pH 7.0 or 6.1.

**Adsorption of DNA at pH 6.1 and 7.0.** The initial interaction between DNA and competent cells which can result in transformation (adsorption) was compared at pH 6.1 and 7.0 to determine whether single-stranded DNA is adsorbed more rapidly than native DNA at the lower pH. Preferential uptake of single-stranded DNA at low pH, as has been observed in the *Haemophilus influenzae* system (5), is not expected since transformation of *B. subtilis* is equally efficient at pH 6.1 and 7.0 with native DNA (Table 2). Rapid removal of single-stranded DNA from solution, however, may provide increased protection from nucleases present in competent culture supernatant fluid (6), thereby enhancing its apparent biological activity. To test this possibility, transformation assays were begun in TME at each pH, and mixtures were diluted 20-fold into identical media at various times to prevent further DNA binding. After a total of 40 min of incubation, cells were plated on selective medium. The results appear in Fig. 4. Native and single-stranded DNA were adsorbed similarly at pH 7.0. Both types of DNA are adsorbed more slowly at pH 6.1. Single-stranded DNA is adsorbed more rapidly than is native DNA at the lower pH, although the same number of transformants was produced in TME at either pH after 40 min of incubation before dilution (Table 2).

**Nuclease activity at pH 6.25 and 7.0.** Incubation of single-stranded DNA with supernatant fluid from competent cultures results in a marked decrease in the transforming activity of the DNA. The inactivating factor appears to be a nuclease(s) with selectivity for single-stranded DNA (6). The following experiment was performed to examine the nuclease activity in supernatant fluids at pH 7.0 and 6.25. Cells were removed from a competent culture of *B. subtilis* 168 by centrifugation. A portion of the supernatant fluid was filtered through a 0.2-μm nitrocellulose filter. A second portion was adjusted to pH 6.25 before filtering. CaCl₂ was added to each portion to give a final concentration of 10⁻³ M. Single-stranded DNA (0.1 μg/ml) was added to the supernatant fluid at each pH. Identical portions of DNA were added to supernatant fluids at pH 7.0 and 6.25 containing 10⁻³ M CaCl₂ and 2 × 10⁻³ M EDTA, as well as to TME containing 10⁻³ M CaCl₂ and TME containing CaCl₂ and an additional 10⁻³ M EDTA. Native DNA was added to supernatant preparations at pH 7.0. After various times of incubation at 37°C, portions of each mixture were diluted 14-fold into TME (pH 5.5) at 4°C. At the end of the sampling period, the diluted mixtures were brought to 37°C, and competent BR151 cells were added. After 40 min of incubation at 37°C, trpC²⁺ transformants were selected. The results are shown in Fig. 5. The biological activity of native DNA is unaffected by preincubation in supernatant fluid at pH 7.0 in the presence or absence of EDTA. In this experiment, incubation of single-stranded DNA in TM alone resulted in the loss of approximately 80% of its biological activity. This loss is presumed to be caused by sticking of the DNA to the glassware and is eliminated by addition of EDTA to the TM. Single-stranded DNA rapidly loses transforming activity on holding in supernatant fluid at pH 7.0. The transformants which appear using DNA incubated 15 min or longer are probably produced by reversibly denatured molecules remaining in the preparation of single-stranded DNA. Inactivation of single-stranded DNA in
supernatant fluids proceeds much more slowly at pH 6.25 than at 7.0. The biological activity of single-stranded DNA is reduced only slightly at either pH when EDTA is added to the preincubation mixture. Preincubation of single-stranded DNA in supernatant fluid which had been boiled for 10 min does not reduce its biological activity beyond the loss caused by holding in TM alone (Fig. 6). It should be pointed out that the transforming activity of single-stranded DNA is reduced by both the cell fraction and the supernatant fluid of competent populations, suggesting that both fractions contain similar nuclease(s). Only the activity of the enzyme(s) which is excreted is investigated in these experiments.

DISCUSSION

The use of the B. subtilis transformation system as a biological assay for single-stranded DNA has been hindered by the low transforming efficiency of this DNA, the sole use of the 168 recipient strain, and the absence of conditions for preferential uptake of single-stranded DNA.

The evidence presented in this paper shows that single-stranded DNA is 50% as active as native DNA in transforming B. subtilis 168 when the pH of the transformation mixture is lowered to 6.1. At this pH, a high efficiency of transformation was observed for five markers distributed in three different recipient strains, indicating that transformation by single-stranded DNA is not limited to one gene or to a particular strain. A decrease in the transforming activity of single-stranded DNA at pH values above 7.0 was observed but was not investigated further.

Both native DNA and single-stranded DNA were found to be adsorbed more slowly to competent cells at pH 6.1 than at 7.0. At the lower pH, however, single-stranded DNA is adsorbed more rapidly than helical DNA molecules. Because of this difference, preferential binding of single-stranded DNA can be achieved by early dilution of the transformation mixture into an identical
medium, although fewer transformants are produced.

Chilton and Hall (3) have shown that the frequency of transformation by native DNA is reduced in the presence of EDTA. This compound appears to limit DNA uptake by removing magnesium which is required during transformation. Evidence presented here demonstrates that magnesium is also required during transformation by single-stranded DNA. The frequency of transformation by native and single-stranded DNA in TME is higher at pH 6.1 than 7.0. If this higher activity is due solely to a lesser capacity of EDTA to bind magnesium at the lower pH, addition of magnesium to TME at pH 7.0 should increase the transformation frequency. No change in frequency would result from the same addition to TME at pH 6.1. This was in fact the case when transformations were performed with native DNA in the presence of additional magnesium. The increased transformation frequency with native DNA at pH 6.1 may, then, be due to a higher level of available magnesium. However, addition of magnesium to TME at either pH did not increase transformation with single-stranded DNA. Thus, a higher transforming activity of this DNA at pH 6.1 cannot be explained solely by the presence of more available magnesium.

Nuclease activity against native DNA was not detected at pH 7.0 or 6.1, but preincubation of single-stranded DNA in supernatant fluid at pH 7.0 resulted in a rapid loss in biological activity. This rapid decrease in activity indicates a dependence on high-molecular-weight DNA for transformation. Such a dependence has been reported by Chilton and Hall (3). The nuclease activity was greatly decreased at pH 6.1 and was eliminated by boiling the supernatant fluid or adding EDTA to the preincubation mixture. No loss of transforming activity was found after preincubation of native DNA in boiled supernatant fluid, indicating that the heat-activated endonuclease reported by McCarthy and Nester (4) was not detectable under the conditions employed. Lowering the pH of TME, therefore, appears to enhance transformation with single-stranded DNA by reducing the activity of a single-strand specific nuclease present in competent cultures of *B. subtilis*. A single-strand specific nuclease has been purified from the supernatant fluids of competent cultures, partially characterized, and shall be reported upon elsewhere.

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**LITERATURE CITED**