Single-Stranded Fraction of Deoxyribonucleic Acid from Bacillus subtilis

MAGDALENA PIWNICKA, DOROTA MACIEJKO,† AND MIROSŁAWA PIECHOWSKA

Institute of Biochemistry and Biophysics, Academy of Sciences, 02-532 Warszawa, Poland

Received 12 November 1980/Accepted 9 April 1981

About 13% of the deoxyribonucleic acid (DNA) of various strains of Bacillus subtilis, independent of the stage of growth or competence for transformation, was rendered acid soluble by endonuclease S1. In a pH 11.2 CsCl gradient, 4% of the untreated DNA banded at the density typical for single-stranded molecules, whereas 9% of the remaining DNA (main band) was sensitive to endonuclease S1. Selective inhibition of DNA polymerase III, or of DNA-dependent ribonucleic acid polymerase, did not increase or abolish single-strandedness. The DNA purification procedure did affect the level of single-stranded DNA, indicating its binding to cell constituents containing ribonucleic acid, protein, and membranous material. The molecular weight of the single-stranded fraction resembled that of total denatured DNA, and its buoyant density in an alkaline CsCl gradient was centered partially at a density of 1.772 g/cm³ and partially at a density of 1.759 g/cm³. Incubation of DNA under conditions leading to renaturation of its single-stranded fraction led to an increase in transforming activity for the purA16" marker (close to the origin of replication) relative to leu-8" and metC3" markers (located in the middle of the chromosome), indicating this region is the main source of the single-stranded fraction.

Single-stranded fractions have been detected in double-stranded, so-called native DNAs isolated from viruses, bacteria and animal cells. Different investigators ascribe the source of single-stranded chromosomal DNA fragments to DNA replication, recombination, or transcription processes (reviewed in reference 21). However, neither the origin nor the role of this fraction has been conclusively elucidated. There is even some doubt as to whether single-stranded DNA exists in vivo or is merely an artifact of DNA isolation procedures (19, 20, 31). This is underlined by the discrepancies in results of different investigators, viz., (i) a correlation of single-strandedness with the competent state or rec" mutation reported by Harris and Barr (11) and LeClerc and Setlow (12) and the lack of such a correlation found by Deddish and Ravin (8) and us (23); (ii) the increase in single-strandedness after phenol treatment observed by Paetkau et al. (20) and us (23), and its disappearance due to phenol treatment, as observed by Deddish and Ravin (8); and (ii) an increase in single-strandedness due to inhibition of RNA synthesis by rifampin found by Deddish and Ravin (8) and a decrease found by us (see below).

Apart from the above, the proportion of single-stranded fragments found by different investigators differs, even in the case of DNA isolated from the same source. For DNA isolated from Bacillus subtilis, Harris and Barr (11) found about 5%, and we (23) found about 15%, single-strandedness. This unusually large single-stranded fraction prompted us to undertake a more detailed study of its origin and characteristics.

MATERIALS AND METHODS

Strains. B. subtilis 168", B. subtilis 168 thy, and B. subtilis 168 thy trp strains were used for preparation of purified lysates and DNA. B. subtilis BUL 717 purA16 leu-8 metC3, kindly supplied by U. N. Streips of the University of Louisville, Louisville, Ky., was used as the recipient strain in transformation and as a source of DNA for renaturation experiments. Phage T7, used as a source of DNA, was obtained from S. Bron of the University of Groningen, Groningen, Germany, and was cultivated on Escherichia coli BB provided by W. Szybalski of the University or Wisconsin, Madison.

Preparation of DNA. ³H-labeled DNA was isolated from B. subtilis 168 thy cultivated in Laird growth medium 1 (C. D. Laird, Ph.D. thesis, Stanford University, 1966) supplemented with vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.) to 0.4%, thymidine to 6 μg/ml, and [methyl-³H]thymidine (Amersham Corp., Arlington Heights, Ill.) to a specific activity of 1.4 Ci/mmol. The DNA was purified in a...
CeCl gradient as previously described (22), followed by phenol treatment and dialysis. The resulting activity of the DNA was 5.9 $\times$ 10$^7$ cpn/µg. 14C-labeled B. subtilis DNA, for use as a position marker in CeCl gradients, was prepared in the same manner, but with omission of phenol treatment and from bacteria cultivated in a medium containing [methyl-14C]thymidine (Amersham Corp.) added to a final activity of 60 mCi/ mmol of thymidine. The specific activity of this DNA was 6.8 $\times$ 10$^7$ cpn/µg. 14C-labeled T7 DNA was isolated from phage propagated on E. coli BB in M-9 medium (2) supplemented with 5-fluorodeoxyuridine and [methyl-14C]thymidine to 30 mCi/mmol in a manner analogous to that previously described for preparation of labeled T6 DNA (24). The specific radioactivity of T7 DNA was 10$^8$ cpn/µg.

B. subtilis cultures and preparation of cell lysates. The cells were cultivated in Penassay broth (Difco additive medium no. 3), supplemented with thymidine and amino acids in the case of auxotrophs, for 12 to 13 h at 37°C with shaking. The bacteria were centrifuged at 9,000 $\times$ g for 10 min and washed twice by suspension and centrifugation in Laird growth medium 1, supplemented with vitamin-free Casamino Acids (Difco Laboratories) to 0.4% and, in the case of auxotrophs, also with thymidine and appropriate amino acids to 20 µg/ml. The packed cells were resuspended with a 10-fold dilution in Laird medium 1 supplemented as above and incubated with shaking at 37°C. After 1 h of incubation, [methyl-3H]thymidine was added to a specific radioactivity of 24 mCi/mmol, and incubation was continued to the late logarithmic stage of growth (about 10$^9$ cells per ml). The culture was chilled, and centrifuged, and the cells were washed twice with cold 0.15 M NaCl-0.01 M EDTA (pH 8.2) and resuspended in 0.15 M NaCl-0.01 M EDTA (pH 8.2) in one-tenth the volume of the culture. Cell lysates (called "purified lysates" in this paper) were obtained by the addition of one-tenth volumes of several enzymes and reagents as follows. Lysozyme was first added to 200 µg/ml, and the cells were incubated at 37°C for 30 min. This was followed by addition of Sarkosyl NL (Ciba-Geigy Corp., Greensboro, N.C.) to 1% and heating at 70°C for 20 min. The temperature was decreased to 37°C, RNase I (Worthington Diagnostics, Freehold, N.J.) was added to 20 µg/ml, RNase T1 (Calbiochem, La Jolla, Calif.) was added to 20 U/ml, and incubation was continued for 30 min. This was followed by the addition of pronase (free of nucleases; Calbiochem) to 200 µg/ml and overnight incubation at 37°C. The digested lysates were deproteinized twice with phenol at pH 8.2 and dialysed at 4°C against four changes of 2 liters of 0.15 M NaCl-10$^{-3}$ M EDTA (pH 8.0). Purified lysates contained about 20 to 30 µg of DNA per ml (specific activity, 10$^4$ cpn/µg) and were used for characterization of single-stranded fractions of B. subtilis DNA. In some experiments, several modifications of the procedure were applied, as described below.

Unlabeled DNAs. Transforming DNA used for evaluation of competence of recipient cultures was isolated from B. subtilis 168$^+$ as described previously (26), but Sarkosyl NL was added to 1% instead of 0.25% during lysis of cells. B. subtilis BUL717 DNA used for renaturation experiments was isolated in the same manner.

Transformation. B. subtilis BUL717 purA16 leu-8 metC3 competent cultures were prepared according to Cahn and From (6) with additions to the media of adenine, leucine, and methionine to 50 µg/ml and cultivation to the density of about 10$^8$ cells per ml. For transformation, the recipient culture was exposed to DNA for 20 min at 37°C, followed by 20 µg of DNase I (Worthington Diagnostics) per ml. At the peak of competence, yields of transformants, with saturating DNA concentrations (1 to 2 µg/ml), were as follows: 0.5 to 1% for the ade$^+$ marker, 0.2 to 0.4% for the leu$^+$ marker, and 0.2 to 0.4% for the met$^+$ marker. Transforming activity of DNA of purified lysates was measured at a concentration corresponding to the linear region of the dose-response curve (0.1 to 0.5 µg/ml).

Endonuclease S1 assay for single-stranded DNA. The endonuclease S1 was assayed at the minimal concentration of the enzyme which digested 97% of heat-denatured B. subtilis DNA under conditions described by Vogt (30). The procedure was as follows. A 20-µl purified lysate sample or diluted solution of purified DNA was added to 10 100-µl samples of the endonuclease S1 reaction mixture with final concentrations of 30 mM acetate buffer (pH 4.6), 1 mM MnSO$_4$, 0.06 M NaCl, and 20 µg of heat-denatured calf thymus DNA (Miles Laboratories, Inc., Elkhart, Ind.). To five samples, 5 µl of endonuclease S1 (Miles) solution was added to a final concentration of 927 U/ml (3 µg of protein per ml), to another five samples, 5 µl of solution without enzyme was added, and all samples were incubated at 37°C for 30 min. Acid-soluble radioactivity was measured by mixing with 25 µl of native calf thymus DNA solution (1 mg/ml) and by the addition of one volume of 10% ice-cold trichloroacetic acid. After 30 min on ice, the samples were centrifuged at 17,000 $\times$ g for 20 min, 250 µl of supernatant was added to 5 ml of scintillator (7 g of 2,5- diphenyloxazole [PPO] plus 0.5 g of 1,4-bis[5-phenyloxazolyl]benzene [POPOP] per liter of toluene)-Tri- ton X-100 mixture (2:1), and 200 µl of water was added for solubilization. Trichloroacetic acid-soluble radioactivity was the difference in counts per minute of samples with and without endonuclease S1 and related to the total radioactivity counted in the sample with DNA solution. The counts in non-precipitated lysate were corrected by the addition of 9% because of quenching. In some experiments (noted below), acid-insoluble radioactivity after endonuclease S1 digestion was measured by spotting samples on Whatman GF/C filter paper disks and precipitating with trichloroacetic acid as previously described (23). This assay was used only occasionally because of precipitation of endonuclease S1 digestion products with the enzyme in the reaction mixture.

Radioactive counting. Radioactive counting was performed as previously described (24).

Thermal denaturation of DNA and CeCl pH 8.0 and pH 11.2 density gradient fractionations. Thermal denaturation and density gradient fractionations were done as previously described (26). The amount of DNA used for fractionation never exceeded 7 µg, to assure adequate resolution of bands.
Denaturing, alkaline CsCl density gradients. Denaturing, alkaline CsCl gradients were prepared by the addition of dry CsCl to 250 μl of DNA solution in 0.15 M NaCl-10⁻³ M EDTA (pH 8.2)-3.0 ml of 0.4 M KPO₄ (pH 12.7), as described by Vinograd et al. (29).

Sucrose gradients. Gradients (5 to 20%) were made up in 0.02 M Tris-0.02 M EDTA-0.9 M NaCl (pH 7.8) solutions. Alkaline gradients contained NaOH, added to a final concentration of 0.15 M. About 0.1 to 0.3 ml containing not more than 1 μg of native or denatured (in 0.25 M NaOH) DNA was layered on the gradient and centrifuged in an SW50.1 rotor at 35,000 rpm for 120 min at 20°C. Collection of fractions and measurements of radioactivity were as previously described (24). The molecular weight of B. subtilis [³H]DNA was calculated as described by Burgi and Hershey (5) from the distance migrated relative to T7 [¹⁴C]DNA, the marker. The molecular weight of T7 DNA was 23.0 × 10⁶, determined by sedimentation in a Spinco model E ultracentrifuge at 17,000 rpm (27).

Inhibitors. A specific inhibitor of DNA replication in gram-positive organisms, viz., 6-[(p-hydroxyphenylazo)uracil] (HPUra) (4), kindly supplied by B. Langley of Imperial Chemical Industries (Alderley Park, Cheshire, England), was used as a stock solution of 5 mg per ml of 0.06 N NaOH. The inhibitor of DNA-dependent RNA polymerase, rifampin (Boehringer Mannheim Corp., New York, N.Y.), was a stock solution of 2 mg per ml of ethanol.

Renaturation. Renaturation was performed at concentrations of 15 to 20 μg of DNA per ml of 0.4 M NaCl-10⁻³ M EDTA (pH 8.0). Samples were incubated at 65°C for 3 h, and the temperature was decreased to 25°C over a period of 2 h. In most experiments, denatured B. subtilis BULT717 DNA was added to 15 or 20 μg per ml of the sample of purified lysate, or purified DNA was added, which itself contained about 20 μg of native DNA per ml. The single-stranded fractions collected from CsCl density gradients (see below) contained 2 μg of DNA per ml of sample, to which 15 μg of denatured B. subtilis BUL DNA was added.

Enzyme digestion of trichloroacetic acid-precipitable radioactivity of purified lysates and analysis of products. A 160-μl sample of purified lysate, containing 10 μg of B. subtilis 168 thy trp DNA and total [³H]-radioactivity of 1.6 × 10⁶ cpm, was used for digestion. Tris buffer (pH 7.6) was added to 0.02 M, MgCl₂ was added to 0.02 M, CaCl₂ was added to 5 × 10⁻⁴ M, DNase I ( Worthington) was added to 20 μg/ml, and the sample (total volume, 188 μl) was incubated at 37°C for 30 min. This was followed by the addition of pH 9.0 Tris buffer to 0.04 M and of 0.4 U of snake venom phosphodiesterase (VPH OEC, Worthington) to a total volume of 248 μl. After 1 h of incubation at 37°C, 2 μl (0.3 U) of alkaline phosphatase (BAPSF, Worthington) was added, and incubation was continued overnight. The digest was reduced to a 20-μl volume over P₂O₅ at room temperature, uridine and thymidine (40 μg each) were added, and the sample was fractionated by chromatography on Whatman no. 1 paper with water-saturated butanol. The large spots of uridine and thymidine were cut out from the dried chromatogram and eluted for rechromatography. The rest was divided into 2-cm² pieces and counted in scintillation fluid.

Trichloroacetic acid-precipitable radioactive activity resistant to alkali. Trichloroacetic acid-precipitable radioactivity was measured by using five 25-μl samples of [³H]thymidine-labeled purified lysate (4,000 cpm each), to which 25 μl of 0.8 N NaOH was added, followed by 1 h of incubation at 37°C and the addition of 50 μl of 0.4 N HCl. Samples (75 μl each) of the reaction mixture were spotted on GF/C filters, dried, and washed with 5% trichloroacetic acid. Their radioactivity was counted and compared with trichloroacetic acid-washed analogous 25-μl samples of lysate to which were added 25 μl of water plus 50 μl of 0.4 M NaCl.

RESULTS

The single-stranded fraction of native B. subtilis DNA was determined by digestion with endonuclease S1, the enzyme specific for denatured molecules (9), and by fractionation on CsCl density gradients at pH 11.2, which increases resolution of native and denatured DNA (29). The results are presented in Table 1 and Fig. 1.

When [³H]-labeled DNA of six representative purified B. subtilis 168 thy trp cell lysates was digested with endonuclease S1, 11.5 to 16.4% (mean 13.8 ± 0.4%) of the radioactivity became trichloroacetic acid soluble. Under the same conditions, endonuclease S1 solubilized about 97% of denatured purified B. subtilis labeled DNA, digested separately or in the presence of purified lysate. In separate control experiments, endonuclease S1 showed no activity against [¹⁴C]-labeled T7 DNA, even when it was first added to B. subtilis cells and subjected to the procedure for preparation of purified lysates. This specificity was observed by measurement of the trichloroacetic acid-soluble fraction but more detailed analysis, by sedimentation on neutral and alkaline sucrose (data not shown), revealed that

<table>
<thead>
<tr>
<th>TABLE 1. Sensitivity of native and denatured DNA to endonuclease S₁.</th>
<th>Trichloroacetic acid-soluble fraction after enzymatic digestion (% of total)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native [³H]DNA in purified lysates of B. subtilis cells</td>
<td>13.8 ± 0.4</td>
</tr>
<tr>
<td>Denatured, purified [³H]- or [¹⁴C]DNA of B. subtilis</td>
<td>97.3 ± 0.8</td>
</tr>
<tr>
<td>Native T7 DNA</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Native T7 DNA treated as was B. subtilis DNA in lysates</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

* Percent of total ± standard error.
VOL. 147, 1981
SINGLE-STRANDED FRACTION OF DNA
209

FIG. 1. pH 11.2 CsCl density gradient fractionation of [3H]thymidine-labeled B. subtilis 168 thy trp cell lysates treated with phenol. (A) Typical, purified lysate; (B) as in (A), but the lysate was heated at 56°C with Sarkosyl; (C) as in (A), but the lysate was heated at 37°C with Sarkosyl. Arrows indicate the position of denatured B. subtilis [3H]DNA, added as a marker and not shown on the graph.

native T7 DNA incubated with endonuclease S1 suffered about three breaks per double-stranded molecule and about seven breaks per single chain.

To determine how much of the radioactivity of our [3H]thymidine-labeled purified lysates could be ascribed to the DNA itself, treatment with alkali was used, and it showed a 3 ± 2% decrease of trichloroacetic acid-precipitable radioactivity in the sample of lysate exhibiting 12 ± 0.2% sensitivity to endonuclease S1. Furthermore, when another sample of the lysate was successively digested with DNase I, snake venom phosphodiesterase, and alkaline phosphatase, 87% of its radioactivity moved from the starting point on a paper chromatogram, and 99% of it moved to the position of thymidine, but only 1% moved to the position of uridine.

When the purified lysates of B. subtilis 168 thy trp cells were fractionated on pH 11.2 CsCl density gradients, about 4% of the DNA formed a band at a density higher than that of the main band and characteristic of denatured molecules (Fig. 1A). This "dense" fraction decreased to 1.9 and 1.7%, and the resolution of bands was poorer when the typical procedure for preparation of lysates was slightly modified by lowering the temperature of heating with Sarkosyl from 70 to 56 or 37°C (Fig. 1B and C).

To correlate these two methods of evaluation of single-stranded DNA content, we measured DNA sensitivity to endonuclease S1 in the lysates before and after CsCl fractionation. The results presented in Table 2 show a correlation of results, but the dense band represented two- to threefold smaller amounts of DNA than that sensitive to endonuclease S1 in the lysate. After collection of DNA from the CsCl gradient, in an experiment identical to that presented in Fig. 1A, the dense band was totally sensitive to endonuclease S1, and about 9% of the main band was still sensitive to the enzyme. The sum was in accord with the about 12% sensitivity to endonuclease S1 found in DNA of the lysate before fractionation on a CsCl gradient.

Origin of single-stranded fraction. Sensitivity to endonuclease S1 digestion was not a characteristic only of DNA isolated from B. subtilis 168 thy trp, but also of DNA isolated from B. subtilis 168 thy cells and B. subtilis 168* cells, independent of the stage of growth of the culture.

A possible relationship between the presence of the single-stranded fraction and RNA or DNA synthesis was investigated with the use of specific inhibitors. Rifampin, an inhibitor of DNA-dependent RNA polymerase, was added to a concentration of 10 μg per ml of [3H]thymidine-labeled B. subtilis 168 thy trp culture in Laird growth medium 1, in the middle of the logarithmic growth phase. An equal volume of the solution without the antibiotic was added to the control culture. The concentration of rifampin to inhibit DNA-dependent RNA synthesis in B. subtilis was based on the investigation by Calvori et al. (7) and controlled by inhibition of
growth of the culture. After 20 and 40 min of incubation, samples were taken for preparation of purified lysates and for determination DNA sensitivity to endonuclease S1, which showed a decrease by one-third due to incubation with rifampin (Fig. 2).

In an analogous experiment, HPUrA, a specific inhibitor of DNA polymerase III, was added to 300 μM, a concentration that inhibits the incorporation of [3H]thymidine into cellular DNA (expressed as a decrease in the amount of [3H]DNA to half that of the control), after 30 min of incubation, corresponding to one cell division. Samples of cultures, with and without inhibitor, were taken for the preparation of purified lysates. Their DNA sensitivity to endonuclease S1 was practically the same and did not show any influence of HPUrA (Fig. 2).

Effect of modifications of treatment of the cell lysate on detectability of the single-stranded fraction of DNA. Several modifications of the procedure for preparation of purified lysates were examined. Their influence on the level of the single-stranded fraction threw some light on its origin and association to different cellular constituents. All experiments presented in this section were performed with the B. subtilis 168 thy trp strain. Omission of RNases and phenol treatment from our typical procedure produced lysates which were not sensitive to endonuclease S1, but subsequent treatment with RNases or phenol revealed the sensitive fraction. In contrast to it, differentially labeled purified denatured B. subtilis DNA, added to cells before lysis, was fully sensitive to endonuclease S1, independent of the procedure for preparation of lysates.

The temperature of heating of lysates with 1% Sarkosyl also critically affected the amount of single-stranded DNA detected. Heating at temperatures lower than 70°C gave DNA of a lower sensitivity to endonuclease S1, but heating at 75°C gave DNA of the same sensitivity as 70°C lysates (Table 3). It should be added that a 56°C lysate, added to nonlabeled cells and subjected to the procedure for preparation of 70°C lysates, did not exhibit increased sensitivity of DNA to endonuclease S1. Furthermore, heating at 70°C, but in the presence of 4 M NaCl (instead of 0.15 M), decreased DNA sensitivity to endonuclease S1 to one-third that of the control (Table 3). In this last experiment, RNase digestion was omitted during preparation of the lysate, and digestion with pronase was performed with simultaneous dialysis against 0.15 M NaCl-0.01 M EDTA (pH 8) to remove excess salt and was repeated with a new portion of the enzyme added after dialysis. The lysate was treated with phenol as usual but was also subjected to fractionation in a pH 8 CsCl density gradient to eliminate any RNA contaminants. DNA collected from the gradient was dialyzed first against 0.15 M NaCl-0.001 M EDTA (pH 8)-0.1% Sarkosyl and then against salt-EDTA solution. Detergent was added to the first dialysis to prevent adsorption of single-stranded DNA on the dialysis bag at high CsCl concentrations. In contrast to experiments described above, the single-stranded fraction could be detected by pH 11.2 CsCl density gradient fractionation, even in cell lysates not treated with RNase, pronase, or phenol, the effects of which were partially reproduced by the mild alkaline pH and the high CsCl concentration. However, in this case the dense fraction contained only about 1% of total DNA as compared to the 4% found in a typical purified lysate (Fig. 1A). As in previous experiments, the separation of dense DNA was poorer, and the amount of banding in a CsCl gradient decreased when the temperature of heating of the lysates with Sarkosyl was lowered to 60 or 37°C. Sarkosyl concentration also appreciably affected the amount of dense DNA in

![Fig. 2. Influence of metabolic inhibitors (+, with; −, without) on DNA sensitivity to endonuclease S1.](http://jb.asm.org/)
the gradients. An increase in Sarkosyl concentration from 0.2 to 1 and 2% and heating at 60°C increased the dense fraction from 0.3 to 0.6 and 1.2% of the total, respectively.

Physical characteristics of DNA of purified lysates. The molecular weight and the buoyant density were determined for DNA of a typical purified lysate of *B. subtilis* 168 thy trp before and after fractionation on a pH 11.2 CsCl density gradient under conditions shown in Fig. 1A. The lysate was prepared from cells cultivated in the presence of [3H]thymidine added to a final radioactivity of 170 mCi/mmol of thymidine in the medium. The main band and single-stranded band were collected from the gradient and dialyzed once against salt-EDTA solution supplemented with 0.1% Sarkosyl to avoid adsorption of single-stranded DNA to the dialysis bag at high CsCl concentrations. This was followed by three dialyses against salt-EDTA solution. The lysate and its DNA fractions thus obtained were sedimented on neutral and alkaline sucrose, leading to DNA bands on similar heterogeneity embracing 25% of the gradient at the half-width of the bands (Fig. 3). Their molecular weights, relative to T7 DNA, are listed in Table 4 and show a decrease from 41 × 10^6 to 25 × 10^6 for DNA fractionated on a CsCl gradient, most likely due to shearing of molecules during collection of fractions. The DNAs of the lysate and of the main band show about two breaks per single chain, by analysis on alkaline sucrose. The molecular weight of the single-stranded band is about 4 × 10^6 and is very similar to that of alkali-denatured DNA of the main band.

Buoyant densities of the same DNA samples, unfractionated and fractionated on pH 11.2 CsCl gradients, were determined in an alkaline (pH 12.7) CsCl density gradient. Differentially labeled denatured T7 DNA (ρ = 1.7720 g/cm³) and denatured unfractionated *B. subtilis* DNA (ρ = 1.7654 g/cm³) were used to obtain the slope of the gradient. The densities were calculated from those characteristic for native DNAs (25) by the addition of 0.062 g/cm³ due to denaturation and by full alkaline titration of molecules at pH 12.7 (29). The results (Fig. 4) show that the main DNA band exhibited a density of 1.768 ± 0.003, practically the same as that of unfractio-

**TABLE 3. Effect of temperature of heating of cell lysate with 1% Sarkosyl at different salt concentrations on DNA sensitivity to endonuclease SI**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>DNA sensitive to endonuclease SI (% of total)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp (°C)</td>
<td>NaCl concn (M)</td>
</tr>
<tr>
<td>37</td>
<td>0.15</td>
</tr>
<tr>
<td>56</td>
<td>0.15</td>
</tr>
<tr>
<td>70</td>
<td>0.15</td>
</tr>
<tr>
<td>75</td>
<td>0.15</td>
</tr>
<tr>
<td>70</td>
<td>4</td>
</tr>
</tbody>
</table>

* Percent of total ± standard error.

这些结果是从测量氯化铁酸可溶性放射性得出的。

**Fig. 3. Neutral and alkaline sucrose sedimentation of [3H]DNA of the purified lysate (A and B), of the main band (C and D), and of the single-stranded band collected from a CsCl gradient (E). Arrows indicate the position of 14C-labeled T7 DNA, added as a marker.**

Downloaded from http://jb.asm.org on October 28, 2017 by guest
ated *B. subtilis* DNA. The single-stranded fraction forms a heterogenous band and the position of its lightest peak corresponds to a density of 1.759 ± 0.003 g/cm³, indicating 38 ± 3% gamma-plus-cytosine content, assuming that both complementary DNA strands are present in this fraction.

**Transformation experiments.** To characterize the chromosomal region from which the single-stranded fraction originated, the transforming activity of three differentially located markers was measured in different preparations of DNA containing different amounts of single-stranded fragments. DNA of purified lysates of *B. subtilis* 168 thy trp cells, heated at different temperatures with Sarkosyl (Fig. 1), and with different sensitivities to endonuclease S1 (Table 5), was used to transform a *B. subtilis* BUL717 ade leu met recipient culture.

The activity of the ade" marker, relative to leu" or met", was higher in the 70°C lysate than in 56 or 37°C lysates and correlated with the amount of single-stranded DNA found in the lysates (Table 5). Furthermore, the specific activity of all markers was lower in 70°C, as compared to 56°C, lysates, but this difference was less pronounced for the ade" marker than for leu" or met" (Table 6). The decrease in specific activity of DNA could be due to more frequent breakage of molecules at 70°C than at 56°C, with inactivation of the ade" marker occurring to a lesser extent than inactivation of leu" or met".

Submission of the lysate to renaturing conditions did not change its sensitivity to endonuclease S1 or the relative activities of the markers (Table 7, no. 1 and 2). Furthermore, the specific activity of DNA in the lysate did not change after heating under renaturing conditions (data not shown). Assuming that the low concentration of single-stranded molecules accounted for the lack of renaturation, we repeated the experiment but with the addition of denatured nonlabeled DNA isolated from the recipient BUL717 strain to a 1:1 ratio with the DNA of the lysate to provoke heteroduplex formation.

---

**Table 4.** Average molecular weights of DNA in fractionated lysates

<table>
<thead>
<tr>
<th>DNA of:</th>
<th>Mol wt of DNA (10^6)</th>
<th>Sedimented on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Neutral surose</td>
</tr>
<tr>
<td>Purified lysate</td>
<td>41</td>
<td>6</td>
</tr>
<tr>
<td>Main band collected from CsCl pH 11.2 gradient</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Single-stranded band collected from CsCl pH 11.2 gradient</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.** Activity of markers and sensitivity to endonuclease S1 of DNA in different, purified lysates

<table>
<thead>
<tr>
<th>Lysate heated with Sarkosyl at temp (°C)</th>
<th>Relative activity of markers</th>
<th>DNA sensitivity to endonuclease S1 (% of total)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ade&quot;/ leu&quot;</td>
<td>ade&quot;/ met&quot;</td>
</tr>
<tr>
<td>37</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>56</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>70</td>
<td>3.7</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*Percent of total ± standard error.

---

**Figure 4.** Relative buoyant densities on pH 12.7 CsCl density gradient of DNA employed; (A) ^14_C-labeled denatured T7 DNA and ^3_H-labeled *B. subtilis* DNA of purified lysate; (B) main band; and (C) single-stranded band, collected from a pH 11.2 CsCl gradient.
This led to renaturation, expressed by a dramatic decrease in sensitivity to endonuclease S1 from 12 to 2%. The relative activities of the markers changed at the same time, with a twofold increase of ade* to leu* (or met*) and a practically unchanged ratio of met* to leu* (Table 7, no. 3). A similar increase in relative activity of the ade* marker was also observed when DNA of the main band, collected from a pH 11.2 CsCl gradient, was submitted to renaturing conditions in presence of BUL717 DNA. Much less pronounced was the influence of renaturing conditions on the relative activities of markers in the single-stranded band from the pH 11.2 CsCl gradient (Table 7, no. 6 and 7). Specific transforming activities of the ade*, leu*, and met* markers of this fraction were 2.3, 1.6, and 1.8%, respectively, of the activities of nonfractionated DNA. This is most likely due to overlapping of about 0.08% of the main band DNA with the single-stranded band containing about 4% of the total DNA of the lysate and is supported by the fact that single-stranded molecules exhibit no transforming activity in the B. subtilis system (1), whereas heating of the single-stranded band under denaturing conditions decreased its activity to nondetectable levels. Renaturing conditions, in the presence of BUL717 DNA, increased the activities of the single-stranded DNA by factors of 2.8 (ade*), 2.4 (leu*), and 1.9 (met*), as compared with the 100-fold increase of ade* activity (0.3 to 30%) on renaturation of the nonfractionated denatured DNA used as a control. Therefore, the influence of renaturing conditions on the activity of single-stranded band is most likely partially masked by the background activity of the overlapping double-stranded molecules.

**DISCUSSION**

About 12 to 14% of the trichloroacetic acid-precipitable radioactivity of purified lysates of B. subtilis cells, labeled with radioactive thymidine, became trichloroacetic acid-soluble after digestion with endonuclease S1. Under the same conditions, 97% of thermally denatured B. subtilis DNA was solubilized by the enzyme, but native T7 DNA was practically unaffected (Table 1). The 13% endonuclease S1-sensitive radioactivity of purified lysates must have originated from DNA, since about 99% of the total label exhibited the density in CsCl gradients, and the

**TABLE 6. Transforming activity of DNA of different lysates**

<table>
<thead>
<tr>
<th>Lyssate heated with Sarkosyl at temp (°C)</th>
<th>Transformants (10^3) per 0.1 μg of DNA</th>
<th>Ratio of 70°C to 56°C lyssate transformants ade*/leu* met* ade*/leu* met*</th>
<th>Ratio of 70°C to 56°C lyssate transformants ade*/leu* met* ade*/leu* met*</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>683</td>
<td>329</td>
<td>391</td>
</tr>
<tr>
<td>70</td>
<td>376</td>
<td>104</td>
<td>150</td>
</tr>
</tbody>
</table>

**TABLE 7. Influence of renaturation conditions on relative activity of markers and sensitivity of DNA to endonuclease S1**

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>Relative activity of markers ade*/leu*</th>
<th>ade*/met*</th>
<th>met*/leu*</th>
<th>DNA sensitivity to endonuclease S1 (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Purified lysate</td>
<td>3.8 ± 0.3*</td>
<td>3.1 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>12.2 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>Renatured lysate</td>
<td>2.8</td>
<td>3.5</td>
<td>0.8</td>
<td>12.7 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>Renatured lysate with BUL DNA</td>
<td>6.7</td>
<td>5.6</td>
<td>1.3</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Ratio</td>
<td>Renatured lysate with BUL DNA/purified lysate</td>
<td>1.8</td>
<td>1.8</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Main band</td>
<td>4.4</td>
<td>3.7</td>
<td>1.2</td>
<td>8.4 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>Main band renatured with BUL DNA</td>
<td>8.1</td>
<td>11.3</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Ratio</td>
<td>Main band renatured with BUL DNA/main band</td>
<td>1.8</td>
<td>3.1</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Single-stranded band</td>
<td>3.8</td>
<td>4.6</td>
<td>0.8</td>
<td>82.0 ± 1.7</td>
</tr>
<tr>
<td>7</td>
<td>Single-stranded band renatured with BUL DNA</td>
<td>4.5</td>
<td>6.7</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Ratio</td>
<td>Single-stranded band renatured with BUL DNA/single stranded band</td>
<td>1.2</td>
<td>1.4</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

* Data are given ± standard error.
resistance to alkali characteristic of DNA, and about 99% of the label of migrating products of nucleolytic digestion of the lysate migrated with thymidine on paper chromatography.

In contrast to the high dose of single-strand-edness detected by endonuclease SI digestion, pH 11.2 CsCl density gradient fractionation of purified lysates showed only 4% of DNA banding at the position of denatured molecules (Fig. 1A). The results of the two methods were reconciled by the finding that the main DNA band from the gradient still conserved partial sensitivity to endonuclease S1. The sum of sensitivities to endonuclease S1 in the two DNA bands corresponded to the sensitivity of the unfractionated lysate (Table 2).

The specificity of our assay for the secondary structure of DNA is supported by the fact that the sensitivity to endonuclease S1 decreased from 12 to 2% after incubation of purified lysate under conditions which renature DNA (Table 7).

The single-stranded fraction is not dependent on the bacterial strain used (see above) or the phase of growth (23). The use of specific inhibitors showed that the level of the single-stranded fraction of DNA was not affected by inhibition of DNA polymerase III by HPUra and decreased by one-third when elongation of RNA chains by DNA-dependent RNA polymerase was inhibited with rifampin (Fig. 2).

These observations, taken together, do not indicate a dynamic function of chromosomal DNA, but suggest rather some static characteristic of the chromosome as a source of most of the single-stranded fraction. If this were the case, most of the single-stranded, or potentially single-stranded, DNA should be located in a specific region(s) of the chromosome. In fact, several observations indicate binding of the single-stranded fraction to the cell membrane: its amount, dependent on concentration and temperature of heating of cell lysates with Sarkosyl and also on treatment with RNases and phenol (see above), is consistent with the known characteristics of the complex of the bacterial chromosome with cell membrane involving RNA and protein (28, 32). Since the best documented case involves complexing of the origin of replication of the B. subtilis chromosome (13, 28), it appeared logical to examine the single-stranded fraction for transforming activity of markers close to the origin, relative to those located approximately midway between the origin and the terminus. Since single-stranded DNA is normally inactive in B. subtilis transformation, but renatured DNA exhibits an activity similar to native molecules (1, 18), purified lysates were subjected to renaturing conditions. Such treatment, but only in the presence of denatured DNA (unlabeled and without the investigated markers), led to a dramatic decrease in sensitivity to endonuclease S1 and about a twofold increase of the purA16" marker activity, relative to leu-8" or metC3" markers, with unchanged relative activities of leu-8" to metC3". A similar result was obtained by renaturation of the main DNA band isolated after fractionation in a pH 11.2 CsCl density gradient (Table 7). Since the purA16 marker is located close to the origin of replication and the leu-8 and metC3 are middle markers of the chromosome (10), we interpret the results obtained with unfractionated lysates and the main DNA band as showing that the single-stranded fraction derives most frequently from the region close to the origin, but does not exclude a simultaneous partial localization in regions not investigated in our assays. Furthermore, lack of renaturation in the absence of denatured DNA (Table 7) may be due to the low concentration of single-stranded molecules in purified lysates and to their poor accessibility when located in double-stranded DNA chains.

For reasons not clear, the single-stranded DNA band isolated from the gradient and submitted to renaturing conditions exhibited a smaller increase in activity, and a smaller increase in activity of the purA16" marker, relative to leu-8" or metC3", than did nonfractionated DNA of the lysate or of the main DNA band isolated from the gradient (Table 7 and above). A possible interpretation is that only a portion of the DNA in the band corresponding to single-stranded molecules exhibits the characteristics of the remainder of the single-stranded fraction in the main DNA band and that this is only partially representative of its potential transforming activity. Such an interpretation is consistent with our observation that the single-stranded fraction of the lysate behaves as though it originated from at least two sources, viz., a region close to the origin of replication, and regions of active transcription (about one-third of the single-stranded fraction).

The purA16" marker appears to be more resistant to thermal inactivation at 70°C (Table 5), possibly due to protection by binding to the cell membrane.

It is pertinent to inquire whether the single-stranded fraction exists as such in vivo or is an artifactual of the isolation procedure, a problem of long-standing interest (19). Our extreme conditions for isolation of DNA employed in this study do not exceed those of the widely applied method of Marmur (14) for isolation and purification of DNA from various sources. The molecular weight of the single-stranded DNA fraction isolated on a pH 11.2 CsCl gradient was close to
4 × 10⁶, comparable to that of unfractionated DNA after denaturation (Table 4). Good renaturability of the entire single-stranded fraction in unfractionated cell lysates also indicates its high molecular weight. This excludes denaturation due to destabilization of the secondary structure of a particular fraction of DNA because of weak stacking forces in short paired chains, such as may arise in newly synthesized single-stranded DNA fragments (19). The single-strandedness of DNA increased from 4 to 12% when the temperature of heating of cell lysates was increased from 56 to 70°C, but remained practically unchanged when the temperature was further increased to 75°C (Table 3). A temperature of 70°C should not denature B. subtilis DNA in 0.15 M NaCl or even DNA with density of 1.697 g/cm³ (16), the density calculated for native molecules corresponding to the portion of the single-stranded fraction forming the peak of lowest density detected in this work (Fig. 5). Furthermore, phenol treatment was indispensable for reproducible detection of 12% single-strandedness in DNA (23). The foregoing, together with all the observations of this study, indicates that we were dealing with a specific fraction of chromosomal DNA which behaves as bound to protein(s), most probably located in the cell membrane. This fraction could be in a single-stranded form or double-stranded but destabilized and visualized as single-stranded because of breakage, or simultaneous denaturation and breakage, of DNA during isolation from the cellular complex. In the case of incomplete dissociation of the complex, this particular fraction of DNA could be lost during phenol treatment, and this could explain why the 56°C lysate contained a lower amount of single-stranded DNA (Table 3) which did not increase after heating at 70°C in the presence of fresh cellular lysate.

One of our results indicates denaturation of DNA during isolation, viz., a decrease in the amount of the single-stranded fraction when 4 M NaCl was used in place of 0.15 M NaCl during heating of the cell lysate at 70°C (Table 3). However, this could also be due to subsequent phenol treatment because of poor dissociation from membrane components under conditions of enhancement of hydrophobic interactions by high salt.

The results presented here and previously (23) do not clarify the discrepancies between results of other investigators (see above). Most probably, they reflect differences between so-called native DNA investigated in vitro and chromosomal DNA functioning in vivo. Nonetheless, they do indicate some specific structure of the region about the origin and are consistent with the widely held assumption that the organization of this region is unique and different from other regions of the chromosome. This is further supported by the recent report of Ogasawara et al. (18) that novobiocin, a selective inhibitor of DNA gyrase, inhibits replication of almost the entire chromosome, with the exception only of the origin region.

Finally, it has been suggested that the foregoing results are interpretable on the assumption that our B. subtilis strain harbors a phage containing single-stranded DNA similar to phage φ1 of E. coli and this it is this DNA which is isolated in our extraction procedure. However, our measurement showed that the amount of DNase-resistant, trichloroacetic acid-precipitable radioactivity in the supernatant is at least 10-fold lower than that expected on the assumption of the existence of a hypothetical phage and about sevenfold lower than that in the single-stranded fraction found in the DNA extracted from the cells. We are consequently justified in concluding that the single-stranded fractions examined in this study are of bacterial origin.

ACKNOWLEDGMENTS

We are grateful to David Shugar for valuable discussions and his help in the preparation of the manuscript. M.P. thanks M. Oishi for critical discussion of the results.

This investigation was supported by the Polish Academy of Sciences (project 09.7.1).

LITERATURE CITED


