Physical Heterogeneity Among *Bacillus subtilis*

Deoxyribonucleic Acid Molecules Carrying Particular Genetic Markers

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In a *Bacillus subtilis* deoxyribonucleic acid (DNA) preparation, extracted and purified by the Marmur procedure (19), it is of general interest to know whether this fragmentation occurs at random or whether there are, instead, unique break points, such that the breakage occurs always and only at these points. The DNA fragments within a particular preparation vary widely in molecular weight, with the transforming activity tending to associate with the higher molecular weight DNA (3, 25). This is not necessarily inconsistent with unique break points, since such break points need not occur at equal distances. However, the unique break point concept requires that, among molecules carrying a particular marker, there be no variation in molecular weight or other physical characteristics. This paper shows that, to the contrary, *B. subtilis* DNA molecules carrying the same marker can vary widely in sedimentation velocity and melting temperature and, to a lesser extent, in CsCl buoyant density. The latter two characteristics have frequently been used to distinguish DNA fragments carrying one genetic marker from those carrying another marker (11, 14, 29).

This understanding of the heterogeneity of *B. subtilis* DNA can be useful in interpreting the results of various transformation experiments. One such experiment, presented here, investigates the effect of acridine orange (AO) on the thermal inactivation of transforming DNA. AO has previously been shown to increase the thermal denaturation temperature of DNA to which it is bound (9, 16). In this paper, it is shown that AO changes the relative thermal stabilities of different genetic markers, and it is suggested that the most reasonable explanation for this effect is one which depends upon our understanding of the size heterogeneity among molecules carrying particular markers.

MATERIALS AND METHODS

Denaturation media. Very dilute saline citrate (VDSC) was composed of 0.003 M NaCl and 0.0003 M sodium citrate, pH 7.2. A mixture of 0.003 M NaCl and 0.0003 M tris(hydroxymethyl)aminomethane (Tris), pH 7.0, was called saline Tris (ST). All pH values were determined at room temperature.

Transformation media. C-1 contained Spizizen’s minimal medium (minimal salts medium without glucose; 31) supplemented with amino acid groups I to V at 6.2 times the concentrations recommended by Lederberg ([17] except 3.1 times for histidine); 0.5% glucose; and 50 μg of uracil per ml, if required by the strain. C-2 contained Spizizen’s minimal...
medium supplemented with 0.5% glucose; 0.02% casein hydrolysate (acid-hydrolyzed and neutralized to pH 7.0); 10 μg/ml each of histidine, phenylalanine, tryptophan, and tyrosine; 0.2 μg/ml each of p-aminobenzoic acid and p-hydroxybenzoic acid; and 10 μg/ml of uracil per ml, if required by the strain. C-3 contained Spizizen’s minimal medium supplemented with 5% glycerol and 0.5% glucose.

**Plating media.** Transformant colonies were scored on plates containing 1.5% agar (Difco) plus 0.5% glucose in Spizizen's minimal medium (31). The appropriate amino acid, purine, or pyrimidine supplements were added to a concentration of 25 μg/ml. Total viable count was scored on plates of Nutrient Agar (Difco).

**Purification of reagents.** The procedure for the purification of AO was described elsewhere (33). CsCl was purchased from the American Potash Chemical Co. and was purified, in 2-lb (907.2 g) batches, by the following procedure: (i) heat overnight at 600 C; (ii) dissolve in 500 ml of water, add 80 g of norite, and stir for 6 to 10 hr; (iii) filter. This solution had an optical density at 260 nm of 0.02 and was concentrated by evaporation to the desired density. *B. subtilis* DNA was prepared by the Marmur procedure (19), followed by banding in CsCl and dialysis against the appropriate solvent. It contained no protein detectable by the test of Lowry et al. (18) and no ribonucleic acid (RNA) detectable by the orcinol test (23). One of the DNA preparations was labeled to a specific activity of 10^6 counts per min per μg by growing the cells in 1 liter of C-1 to which 10 μc of methyl-H-thymidine (New England Nuclear Corp.) had been added. Bacteriophage λ DNA was the generous gift of James Champoux. For DNA solutions, the value of 1 μg/ml was taken to equal 3 × 10^5 M.

**B. subtilis strains.** *B. subtilis* strains included SB-5, his-1 trp-2 ura-1; SB-19, prototroph; SB-738, lys-1; SB-1012,aro-2 trp-2 his-2 tyr-1 his-1 cys-1; SB-1017,aro-2 trp-2 tyr-1 cys-1 his-1 lys-1. The markers aro-2 trp-2 his-2 and tyr-1 are all part of a single linkage group (26). The remaining markers are not linked by transformation.

**Thermal denaturation.** DNA solutions, in volumes ranging from 0.1 to 1.0 ml, were prepared in 2-ml tubes with ground-glass stops, taking care that all of the solution was placed cleanly on the bottom without letting any adhere to the neck where it might escape heating. The tubes were heated in a Haake temperature-controlled ethylene glycol bath for 20 min, removed, and quickly cooled by rapid swirling in an ice-water slurry. The DNA solutions were then assayed for transforming activity. Control experiments showed that the decrease in transforming activity was frequently not complete until 20 min. Heating beyond 20 min caused no significant additional drop in transforming activity. The latter result argues against the possibility that any significant portion of the inactivation is the result of depurination or strand scission (1, 13).

**Transformation.** The procedure for the growth of competent cells was developed by Nester, Schaefer, and Lederberg (26) and was modified successively by W. Bodmer and L. Wang, L. Okun, and C. Laird (personal communication). A 5-ml solution of Penassay Broth (Difco Antibiotic Medium 3), supplemented with 0.5% glucose, was inoculated from either a slant or a single colony, shaken at 37 C for 12 to 13 hr, and centrifuged; the pellet was resuspended in 0.5 ml of C-1. An 0.2-ml amount was added to 20 ml of C-1 in a 250-ml Erlenmeyer flask and was shaken vigorously at 37 C. When the Klett reading indicated that exponential growth had ended, the entire 20 ml was added to 80 ml of C-2 in a 250-ml Erlenmeyer flask and was incubated, with slow shaking, at 30 C for 2 hr. The culture was centrifuged, and the pellet was resuspended in 10 ml of C-3, divided into 1-ml samples, and frozen in liquid nitrogen.

For the transformation assay, competent cells were thawed and diluted (generally 1:10) into Spizizen's minimal salts medium plus 0.02 M MgCl_2. In some experiments, the Spizizen's minimal salts medium was modified by leaving out the potassium phosphate (32) and by adding 0.5% glucose.) The cells were distributed to tubes, DNA was added, and the tubes were shaken at 30 C for 30 to 45 min. Pancreatic deoxyribonuclease was added to 10 μg/ml; the tubes were shaken 10 min more, and the cultures were spread on the appropriate plates.

Competent cells prepared in this way and assayed with saturating levels of DNA (4 μg/ml) routinely gave about 1% transformation frequency. The freezing and thawing process resulted in losses of competence or viable counts, or both, ranging from 0 to 50%.

**Sedimentation.** Zone sedimentation was carried out by the method of Burgi and Hershey (5), except that untreated polyallomer centrifuge tubes were used. Centrifugation was for 3 hr at 35,000 rev/min. Drops were collected with an apparatus of the type made by Hoefer Scientific Instruments, San Francisco, Calif., in which the bottom of the tube is punctured in a controlled manner by a hollow needle. The drops flowed out through the needle at a constant volume, throughout the gradient, of between 13 and 14 μlitters per drop. Seven-drop fractions were collected in disposable glass tubes.

Solutions for CsCl centrifugation contained 0.01 M Tris (pH 8.5), 0.001 M ethylene diamine tetraacetic acid, and sufficient CsCl to give a refractive index of 1.4002 ± 0.0004. They were centrifuged in a Spinco model L with a no. 50 fixed angle rotor, spinning for 66 hr at 32,000 rev/min. The polyallomer centrifuge tubes and Pyrex collection tubes were pretreated by soaking for 24 hr in a 1 mg/ml solution of bovine serum albumin (BSA). The tubes were then allowed to dry without ringing and were used within 48 hr. The BSA treatment was necessary to prevent binding of DNA to the tubes (2). Drops were collected with the same apparatus as that described for sucrose gradients. With CsCl, the drop size was about 9 μlitters and 12-drop fractions were collected.

**Radioactivity counting procedure.** Scintillation counting was done as described by Bodmer and Ganesan (4).
RESULTS

Heterogeneity in sedimentation velocity. A 2.0-μg amount of tritium-labeled SB-738 DNA, prepared as described above, was sedimented through a sucrose gradient, producing a broad, relatively smooth peak. Five fractions from each wing of the peak of transforming activity (leaving six fractions at the center) were pooled and dialyzed against VDSC, and samples of each solution were sedimented in separate sucrose gradients. Figure 1 shows the sucrose gradient sedimentation of solution I (the five fractions from the fast side of the peak) and solution II (from the slow side). The SB-1012 DNA which was added as a standard is auxotrophic for the other markers tested. The difference between the positions of the peaks in the two gradients was approximately that expected on the basis of the fractions taken from the original gradients. Thus, the breadth of the sucrose gradient peak results from real heterogeneity in the size of the molecules, and, by selecting material from opposite sides of the peak, solutions of long (solution I) and short (solution II) molecules have been obtained. (Long and short refer to high and low molecular weight, respectively. Heavy and light refer to high and low buoyant density, respectively.) The standard DNA, sedimented with bacteriophage λ DNA in a separate gradient, was calculated, by the method of Burgi and Hershey (5), to have an average molecular weight of 16.7 × 10^6. [The term "average molecular weight" is intended only to imply the molecular weight calculated from the position of the peak of the activity under analysis. It would not be precisely accurate to call it either a number average or a weight average since, although the transforming activity of a DNA molecule increases with molecular weight (25), there is no evidence for any simple mathematical relationship between the two parameters.] With this as a molecular weight standard, the average molecular weights of the long and short molecules were calculated to be 34 × 10^6 and 13 × 10^6, respectively. The sedimentation behavior of this DNA preparation did not change during its 5 months of use, indicating that the sedimentation pattern was not substantially affected by tritium.

![Figure 1. Sucrose gradients of long and short molecules. Samples (0.1 ml) of solutions I and II were mixed with 5 μl of approximately 10^{-4} M SB-1012 DNA (lys-I^+), which was used as a standard. These solutions were sedimented in separate sucrose gradients. A 20-μl amount of each fraction was assayed for radioactivity, and 50 μl was assayed by transformation of SB-1017. Each transformation mixture contained 1.0 ml, and the graphs show the number of colonies per 0.2 ml of this mixture. (a) Solution I, long molecules; (b) solution II, short molecules. Symbols: ●, lys-I^+ (standard) colonies; □, trp-2^+ colonies; △, his-I^+ colonies; ○, 3H counts per 10 min in (a); 3H counts per min in (b).]
decay or other time-dependent degradative activities.

**Heterogeneity in thermal stability.** It would be expected that such a variation in molecular weight would be correlated with a substantial variation in thermal stability, and this was, in fact, the case. As shown in Fig. 2, the transforming activity of solution II is much more sensitive to heating than is that of solution I.

**Heterogeneity in buoyant density.** Similarly, molecules of high and low buoyant density could be selected from opposite sides of the DNA peak in a CsCl density gradient (Fig. 3). Five fractions from each wing of the peak were pooled to form solution A (heavy fractions) and solution B (light fractions), respectively. Samples of the two solutions were run in separate CsCl gradients (Fig. 4). The lys-I+ activity represented the added standard DNA, auxotrophic for the other analyzed markers. Both the his-I+ and cys-I+

![Fig. 2. Inactivation of long and short molecules. Solutions I and II (long and short molecules, respectively) were diluted so that each was present at $6 \times 10^{-1}$ x in solutions of $1.2 \times 10^{-4} \times$ SB-1012 DNA (auxotrophic for all markers tested) in VDSC. These solutions were heated and assayed by transformation of SB-1017, diluting the DNA 1:100 (long molecules) or 1:20 (short molecules) into the transformation mixture. (Controls without the SB-1012 DNA showed that its effect on the transformation frequency was negligible.) At these dilutions, the unheated DNA of either solution produced about $3 \times 10^5$ transformants per ml. Symbols: $\Delta$—$\Delta$, trp-2+, long molecules; $\bullet$—$\bullet$, his-I+, long molecules; $\triangle$—$\triangle$, trp-2+, short molecules; $\circ$—$\circ$, his-I+, short molecules.

![Fig. 3. Selection of light and heavy fractions. A 24-µg amount of SB-738 DNA (the same DNA preparation as that used for the experiments in Fig. 1 and 2) was sedimented in a CsCl gradient. Twelve-drop fractions were collected, and 5 µl of each was assayed by transformation of SB-1017. Each transformation mixture contained 1.0 ml, and the graph shows the number of colonies per 10 µl of this mixture. Symbols: $\bigcirc$, cys-1+ colonies; $\bigtriangleup$, his-1+ colonies; $\square$, trp-2+ colonies. Solution A was taken from fractions 44 through 48. Solution B was taken from fractions 53 through 57.]

Activities were about one fraction heavier in solution A than in solution B, whereas the trp-2+ position was approximately the same in both. (The density gradient was approximately 0.001 g per ml per fraction.) To avoid cluttering the figure, the cys-I activity is not shown. Its pattern was essentially identical to that of his-I.

To select fractions that were symmetrical with respect to the his-I and cys-I peaks, it was necessary to be asymmetrical in the selection of trp-2 molecules. Therefore, there is much less trp-2 activity in solution A than in solution B).

The average molecular weights of the transforming DNA in solutions A and B were found to be 15 and 18 million, respectively, when analyzed in the same way as solutions I and II (above).

Thus, within a normal B. subtilis DNA prep-
reration, molecules carrying a particular marker are heterogeneous with respect to molecular weight, thermal stability, and buoyant density. This heterogeneity must be taken into account when drawing conclusions from studies of the physical properties of transforming DNA, and it also suggests possible explanations for otherwise poorly understood phenomena in transformation. In particular, I would like to discuss the experimental observations which stimulated this investigation of molecular heterogeneity.

Differential effect of AO on marker inactivation. Figure 5 shows the heat inactivation, with and without $2 \times 10^{-4} \text{ M AO}$, of the transforming activity for the his-1 and trp-2 markers, which are naturally high and low melting, respectively. The inactivation curves were shifted to higher temperatures by the AO, and, as shown in Fig. 6, the maximum $\text{his-1/trp-2}$ ratio was increased approximately fourfold. (The AO concentration was far below that required for inhibition of transformation.) AO increased the thermal stability of the $\text{ura-1}$ marker (not shown here), intermediate in its normal melting characteristics, more than that of $\text{trp-2}$ and less than that of $\text{his-1}$.

(Peaks such as that at 88.5°C in Fig. 5 are frequently observed with denaturation at this low salt concentration. That this peculiarity is not responsible for the differential marker effect of AO is shown by the fact that the same effect is observed when such a peak occurs in the curves without AO and not in the curves with AO).

Differential effect of AO on long and short
molecules. Thus, the natural differences between the thermal stabilities of different markers could be substantially increased by the presence of AO. This increase in the his/trp ratio would be expected if the presence of the AO caused a sharpening of both inactivation curves. Such a sharpening would imply a decreased heterogeneity in melting behavior. Since it is now known that much of this heterogeneity is due to differences in molecular weight, it is a reasonable guess that AO may be reducing the differences between the melting behavior of short and long molecules.

Preliminary experiments, in which long and short molecules were heated with and without $10^{-6}$ M AO, suggest that this is so (Fig. 7). [In these experiments, the DNA was mixed with an excess of SB-1012 DNA (auxotrophic for all markers observed) so that there was the normal mixture of DNA molecules to compete for the AO.] The AO could increase the thermal stability of short molecules more than that of long molecules. This appeared to be true for both markers, although the data were more convincing for his-1. Therefore, the presence of AO in the solution of unfractionated DNA could result in a decrease in the overlap of melting temperatures of long trp-2 molecules and short his-1 molecules, and thus in an increase in the ratio of his-1/trp-2 surviving activities.

**DISCUSSION**

Heterogeneity among molecules carrying the same marker. The results presented here have demonstrated that, within a particular *B. subtilis* DNA preparation, isolated by the standard Marmur procedure, individual markers can be carried on molecules that vary widely in their physical characteristics. This indicates that there are not unique break points, such that breakage occurs always and only at these points. It has
been demonstrated that each of the markers analyzed can be carried on molecules of at least four different molecular weights. This implies at least four break points for each marker, two, three, or four of which are broken in each chromosome. The breadth and relative smoothness of the band of DNA, in the sucrose gradient, suggest that there is actually much more heterogeneity in molecular weight and that, in fact, the breaks occur at random.

This conclusion differs with that of Roger, Beckman, and Hotchkiss (28), who concluded from sedimentation boundary and thermal denaturation experiments that DNA isolated from *Pneumococcus* is of homogeneous molecular weight and that, during preparation, it breaks only at pre-existing weak linkages. Gabor and Hotchkiss (10) gave further evidence for homogeneity in pneumococcal DNA preparations by showing that there was a difference in the time of uptake of different markers, which would not be expected if the molecules were broken randomly so that all markers appeared, with equal frequency at the ends of molecules. Strauss (34) did similar experiments in *B. subtilis*, and his data do not show such a variation, although he does not stress this point in his conclusions. Thus, it is conceivable that the difference between the findings reported here and those of Roger et al. is the result of an intrinsic difference between the DNA of *B. subtilis* and *Pneumococcus*. It is also possible that the difference is due to differences in the DNA isolation procedure. However, it would have to be something more subtle than differences in shearing force or amount of nuclease activity, since the molecular weights of the DNA preparations studied by Roger et al. were intermediate between the extremes of the molecular weights in the present study. Massie and Zimm (22) presented data suggesting that the *B. subtilis* chromosome may consist of subunits, having a molecular weight of $250 \times 10^6$, held together by protein. Since the results presented here deal with molecular weights which are lower by an order of magnitude, there is no contradiction.

**Local compositional heterogeneity.** Both the *his-l* and *cys-l* markers can be carried on molecules of varying buoyant density. Since buoyant density varies with base composition (30), this suggests a local compositional heterogeneity in the chromosomal region of each of these two markers. It is conceivable that the buoyant density differences are the result of minor changes in configuration occurring during DNA isolation. However, it is difficult to imagine a mechanism that is capable of producing such a change but that does not produce equal change in the buoyant density of molecules carrying the *trp-2* markers.

Similar conclusions about local compositional heterogeneity have been drawn for the *Pneumococcus* genome (14, 24, 27). However, since those experiments involved the comparison of different DNA solutions which had been exposed to different shearing forces (rather than selected samples of a single solution), they cannot be cited as evidence against preferential break points.

**Differential thermal resistance of long and short molecules.** The transforming activity of short molecules was inactivated by heat much more readily than the transforming activity of long molecules. This finding was predictable on the basis of the observations of Geiduschek (12) and Crothers, Kallenbach, and Zimm (6); it is also direct confirmation of the conclusions of Guild (14) and of Mindich and Hotchkiss (24) from their observations that shearing increased the sensitivity of transforming activity to heat inactivation. Thus, although it is conceivable that the thermal lability of the short molecules could be due to the absence of a region particularly rich in guanine plus cytosine that confers stability upon the long molecules (an unlikely possibility in view of the similar effects of size differences on the thermal stability of all markers tested), it is much more likely that, when significant differences in molecular weight exist, they are the primary determinants of the differences in thermal stability.

**Differential effect of AO on thermal stability.** Our understanding of the heterogeneity within a DNA preparation makes possible an interpretation of the results of transformation experiments that are otherwise difficult to understand. An example of such a result is the differential effect of AO on the thermal inactivation of various genetic markers.

It should be noted that, because of differences in the AO and DNA concentrations and in the portion of the DNA population whose melting was observed, the experiment described in Fig. 5 and 6 is not directly comparable with that described in Fig. 7. Figure 7 merely shows that the effect of AO on thermal stability can increase with decreasing molecular weight. It does not deal with all of the molecular sizes that are important for the effect seen in Fig. 5 and 6. The increase, with AO, of the *his-l*/trp-*l* ratio in the experiments in Fig. 5 and 6, occurring as it does in the high-temperature region of the curve, can be explained as the result of an AO-induced decrease in the overlap between the melting temperatures of very long *trp-2* molecules and moderately long *his-l* molecules.

There are, however, two alternative explana-
tions that could conceivably account for all or part of the increase in his-1/trp-2. The average guanine plus cytosine content of his-1 molecules is greater than that of trp-2 molecules, as indicated by both $T_m$ (20) and buoyant density (30). If AO binds more strongly to DNA rich in guanine plus cytosine, it would be expected to protect the his-1 molecules more effectively than the trp-2 molecules. Alternatively, it has been shown (33) that under certain conditions AO, which is dissociated from early melting molecules as one consequence of denaturation, can be transferred to still native molecules, which are thus afforded a greater degree of protection. If this occurs, the his-1 molecules would be more effectively protected than the trp-2 molecules. Neither of these explanations seems likely since, in view of the high AO concentrations and low salt concentration, there are probably very few DNA molecules of any type whose strong binding sites are not saturated with AO.

Greater effect of AO on short molecules. The following is suggested as a possible explanation for the greater effect of AO on short molecules. It has been consistently observed (7, 11, 21) that the inactivation of transforming activity takes place at higher temperatures than the hyperchromic transition. Freifelder and Davison (8) showed that successful strand separation did not begin until 75% of the hyperchromicity had been attained and that many of the strands were not separated even after the hyperchromic transition was complete. Inman and Baldwin (15) and Wake and Baldwin (35) showed that physically or enzymatically produced hybrids of dAT: dABU required several hours to achieve complete strand separation, when held at temperatures slightly above the hyperchromic transition zone, in low ionic strength media. These data are consistent with the idea discussed by several authors [e.g., Dove and Davidson (7)] that the inactivation of transforming activity is a two-step process, the first step (that measured by hyperchromicity) being the disruption of the hydrogen-bonded, helical conformation of the molecules and the second step being the actual physical separation of the strands. These two steps are manifestations of, respectively, reversible and irreversible denaturation (12). Thus, completion of only the first step would leave the molecule capable of renaturating, upon cooling, to more or less perfectly restore the helical conformation and thus the transforming activity.

As pointed out above, the first step ordinarily takes place at a temperature considerably lower than that required for the second step. However, when the melting temperature is substantially increased by AO, there is no longer any observable difference between the temperatures of the hyperchromic transition and of the inactivation of transforming activity. [For example, compare the curves in Fig. 5 with the $T_m$ values for the hyperchromic transitions under the same conditions: 58.5°C without AO; 82.7°C with $2 \times 10^{-4}$ M AO (33)]. The most economical interpretation of this phenomenon is that the primary effect of the AO is on the first step of the denaturation; when the AO increases the temperature of the first step to a point above the temperature normally required for the second step, there is no further increase in the temperature of the latter, and, therefore, steps 1 and 2 occur at the same temperature.

Now, long and short molecules, of the molecular weight with which we are dealing, are expected to have very similar hyperchromic transition curves, but the long molecules are expected to require a higher temperature for strand separation (6, 12). This is reflected in the observed increase in the thermal stability of transforming activity with increases in molecular weight. However, when AO causes an increase in the temperature of step 1, so that the hyperchromic transition of both types of molecule occurs above the normal temperature of strand separation for either, both steps would be expected to occur at the same temperature for both types of molecules, and the natural advantage of the long molecules would be eliminated. At low AO concentrations, as in Fig. 7, the temperature of the hyperchromic transition would only be raised enough to reduce, but not to eliminate, the advantage of the long molecules.

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**LITERATURE CITED**