JOURNAL OF BACTERIOLOGY, May 1971, p. 608-614
Vol. 106, No. 2
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Quantitative Aspects of Deoxyribonucleic Acid Renaturation: Base Composition, State of Chromosome Replication, and Polynucleotide Homologies

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Received for publication 10 February 1971

The base composition of a deoxyribonucleic acid (DNA) sample affects its intrinsic rate of renaturation. In agreement with the information of Wetmur and Davidson, it was established that high guanosine plus cytosine (GC) DNA renatures faster than expected from analytical measurement of its molecular weight. A calculated correction factor of 1.8% of the observed $C_0t_{.5}$ is required for every mole per cent GC difference from 51% GC. The correction factor is now established in the range of 32 to 65% GC. Renaturation of DNA mixtures prepared from pairs of organisms has been studied. When no similarity existed between the two organisms, the observed $C_0t_{.5}$ of the mixture was the sum of the independently determined $C_0t_{.5}$ values. Lack of additivity was correlated with similarities in polynucleotide sequence of the reassociating DNA molecules. A quantitative relationship was formulated to relate $C_0t_{.5}$ values of renatured DNA mixtures to per cent binding ("homology"). Finally, it was demonstrated that DNA prepared from log-phase cells renatures faster than stationary-phase DNA and also departs from theoretical second-order kinetics.

In recent years, a number of molecular techniques have been used to assess similarities of nucleotide sequence among groups of procaryotes. The most frequently employed methods involve the reassociation or renaturation of deoxyribonucleic acid (DNA) molecules from two organisms. A new kind of procedure has evolved from the early work of Marmur and his colleagues (19) on the qualitative renaturation studies of viral and bacterial DNA. From this origin, Britten and Kohne (7) illustrated quantitatively an apparent proportionality between the complexity of the DNA (i.e., molecular weight of the genome expressed in daltons) and its $C_0t_{.5}$. The $C_0t_{.5}$ (mole second liter^{-1}) corresponds to the time at which a given concentration of a DNA sample attains 50% renaturation.

It was indicated recently that a knowledge of the genome size of bacteria might be useful in assessing evolutionary relationships and their direction of advancement (16). Furthermore, this knowledge might have useful taxonomic implications. Two bacteria with different genome sizes would most likely not be closely related (11). Optical measurement of DNA renaturation was used by several investigators to ascertain such taxonomic information (1, 4, 11, 15). The results demonstrate that most eubacteria differ in size from the *Escherichia coli* genome by 30% or less. Relatively minor influences on experimental precision would obscure real differences in genome size. Therefore, we believed it appropriate to explore further the effect of base composition and the state of chromosomal replication on renaturation kinetics. A test procedure is provided which can serve as an internal control in defining quantitative limitations of the renaturation technique. A slight variation of this procedure permits quantitative estimates of polynucleotide sequence binding between two renaturing DNA samples.

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MATERIALS AND METHODS

Bacterial cultures and media. *Mycoplasma* lysates were the generous gift of W. H. Kelton. Unless indicated in the text, the enteric bacteria were propagated in Penassay medium (Difco) at 37 C and harvested during the stationary phase of growth. H-I *Bdellovibrio*

109 was grown at room temperature to the stationary phase in PYE broth (23). *E. coli* C, 15 TAU, and K-12 were obtained from J. C. Suit and strain B was from S. E. Luria; D. J. Brenner provided *Shigella flexneri* ATCC 24570; *Salmonella typhimurium* LT2 was acquired from H. H. Plough.

Extraction and purification of DNA. Washed cells were lysed with 2% sodium dodecyl sulfate, and the DNA was extracted and purified by a modification of the Marmur procedure (17). In this modification, deproteinization was accomplished with neutralized saline-ethylenediaminetetraacetic acid equilibrated phenol. After ribonuclease and Pronase treatment, samples were further deproteinized with phenol. Some samples also were treated with 2-methoxyethanol to remove polysaccharide material (3). However, this additional treatment had no detectable effect on the experimental determinations.

Preparation of DNA for renaturation. Several methods of shearing the DNA were investigated. These included sonic oscillation and repeated passage of the DNA through a 27-gauge needle or through a French press at 15,000 psi. Shearing with the French press proved to be the most convenient and reliable method and was employed to shear all DNA samples in this study. Sheared DNA [200 to 300 μg in SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7) per ml] was filtered through a 0.45-μm cellulose nitrate filter (Millipore Corp.) and dialyzed to equilibrium in SSC. After dialysis, the DNA was again filtered and stored over $CHCl_3$ at 4 C. Sheared samples were discarded if not used within 4 days.

Renaturation experiments. The sheared DNA sample, typically 50 to 60 μg/ml, was placed in ground glass-stoppered cuvettes which were sealed with several drops of mineral oil. The DNA was heat-denatured in a thermostated automatic recording spectrophotometer (Gilford Instruments, Inc.). The denaturing temperature was maintained for 6 to 10 min after the completion of the hyperchromic increase at 260 nm and then rapidly (15 to 20 min to equilibration) returned to 25 C below the T_m (T_m −25 C). The optical density after the temperature drop was taken to represent 0% renaturation (although some undoubtedly occurs), and the time was recorded. One hundred per cent renaturation corresponded to the optical density of native DNA at T_m −25 C before the hyperchromic shift.

Renaturation data are plotted in the customary log C_0t versus per cent reassociation, as suggested by Britten and Kohne (7). Our concentration of DNA in moles per liter is based on a sodium nucleotide mean residue weight of 331 daltons (10). A C_0t of 1 results from the incubation of 92.7 μg of DNA for 1 hr and therefore differs slightly from the value used by Britten and Kohne (7).

Genome sizes are estimated from the proportionality relationship between the $C_0t_{.5}$ of a standard of known genome size and the $C_0t_{.5}$ of the unknown. We will show that this proportionality is modified by the guanosine plus cytosine (GC) content of the renaturing DNA.

We used *E. coli* B as a primary molecular-weight standard by using a value of 2.2×10^9 daltons for the genome size. This value is Eigner's interpretation of the molecular weight estimated from Cairns' best autoradiograph of an *E. coli* genome of about 1,100 μm in length (9). In the present study, four molecular-weight standards are used, and this value for *E. coli* is consistent with all standards.

RESULTS

Effect of the base composition of DNA on renaturation kinetics. In their comprehensive quantitative studies on deoxyribonucleic acid renaturation, Wetmur and Davidson (24) mentioned that the GC content of the DNA appeared to have a slight effect on its renaturation kinetics. This phenomenon is demonstrated in Fig. 1 which shows a plot of the relative rate constant and the GC base composition for a variety of DNA samples. The rate constant is established from the quotient of the analytically determined molecular weight (ascertained from microscopic contour measurements or sedimentation of whole phage DNA molecules); the molecular weight was established from reassociation kinetics by using *E. coli* as the reference. Figure 1 illustrates four points from Table 4 of Wetmur and Davidson; DNA of coliphage T4, SV40, *E. coli*, and bacteriophage N1 (24). The remaining four points are from our own observations of DNA of PPLO H-39, coliphages T2 and P1 *vir*, and *E. coli*. To explain further, the value obtained by renaturation kinetics for the genome size of PPLO H-39, which has a GC content of 32%, is too large as compared to the value obtained from contour measurements (836×10^6 from kinetics versus 510×10^6 to 530×10^6 daltons from contour length, Table 1; references 5, 21). The quotient of the two observations (relative rate constant) is about 0.63, and this corrected

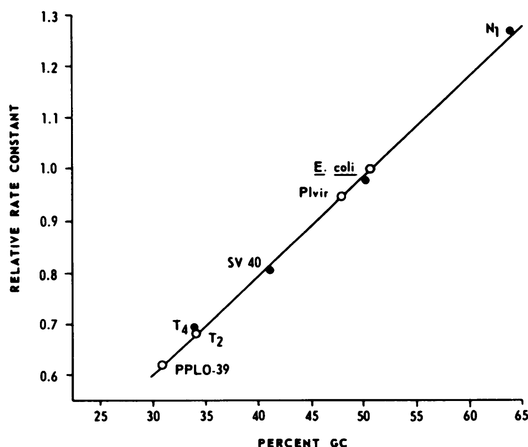

FIG. 1. *Dependence of second-order renaturation relative rate constants upon GC content of DNA. Data of Wetmur and Davidson (24),* ●; *data from this report,* O.

TABLE 1. *Effect of DNA base composition on genome size determined from renaturation kinetics*

Source of DNA	Genome size $\times 10^{-6}$ daltons		
	Observed (kinetics)	Corrected for GC content[a]	Reported[b]
E. coli B	2,200	(51)[a] 1 × 2,200 = 2,200	2,200 (9)
Mycoplasma H-39	836	(32) 0.63 × 836 = 527	510–530 (5, 21)
P1 *vir*	74.8	(48) 0.95 × 74.8 = 70.3	71 (13)
T2	180	(34) 0.7 × 180 = 126	130 (9)
N1	24 (24)[b]	(64) 1.27 × 24 = 30.5	33 (24)
T4	180 (1)[b]	(34) 0.7 × 180 = 126	130 (9)

[a] Guanosine plus cytosine (GC) base composition (per cent) is given in parentheses.
[b] Literature cited in parentheses.

relative rate constant falls directly on the plot established from the original estimates of Wetmur and Davidson (24).

Table 2 demonstrates additional examples of the effect of DNA base composition on genome sizes determined from reassociation. It can be seen that a high degree of agreement between kinetic and analytical estimates of genome size is achieved only when these corrections are applied. When the experiments are carefully controlled, it is possible to demonstrate the need for as small a correction as 5% for a DNA sample of 48% GC (P1 *vir*, Table 2).

An average correction factor (slope of Fig. 1) is 0.018/% ΔGC, relative to the GC content of the standard *E. coli*. Thus, organisms with a GC content of 41 or 61% would require an 18% decrease or increase, respectively, in their $C_0t_{.5}$ for the most precise estimate of their genome size.

An equation which relates these corrections to the observed $C_0t_{.5}$ and the estimated genome size can be described as

$$G = (2{,}200 \times 10^6\ X/Y) \cdot 1 - (\Delta GC_{51} \times 0.018) \quad 1$$

In this equation G is the genome size, Y is the $C_0t_{.5}$ of *E. coli* with a genome size of $2{,}200 \times 10^6$ daltons, X is the $C_0t_{.5}$ of the experimental sample, and ΔGC_{51} represents the % GC difference between the sample and *E. coli* DNA (% GC of *E. coli* − % GC of sample). The entire latter term in equation 1 can be replaced by the relative rate constant (RC) which can be estimated from Fig. 1. In this case, equation 1 would reduce to

$$G = (2{,}200 \times 10^6\ X/Y)\ (RC) \quad 2$$

Renaturation of DNA mixtures. Although Britten and Kohne (7) reported four reference standards in their original work to illustrate the relationship between $C_0t_{.5}$ and genome size (*E. coli* DNA, coliphage T4 DNA, replicative double-stranded MS-2 RNA, and poly U + poly A), they apparently determined only the data for *E. coli* and T4 DNA species by optical methods. Their data indicated about a 20-fold difference in the complexity of these two genomes, similar to the values obtained from analytical measurements, even though there was no correction for the GC effect. We decided to test whether the relationship between genome complexity and $C_0t_{.5}$ would still yield the expected results over more narrow and subtle variations in complexities. A procedure was devised which would serve as an internally controlled examination of the basic assumptions involved in genome size estimates based on renaturation kinetics. The experiments involved renaturing mixtures of two DNA species with no expected sequence homology. It was predicted that the observed $C_0t_{.5}$ value of the mixture of appropriate concentrations would be the sum of the $C_0t_{.5}$ values determined from each independent sample, and the results are independent of any predetermined measurements of genome complexity.

Several of these experiments are shown in Table 2. The preparations consisted of DNA mixtures of *E. coli* and T2 or of *E. coli* and *Bdellovibrio* DNA species. The mixes were prepared in a manner which reflects equivalent amounts of genome equivalents. The data in Table 1 illustrate that the *E. coli* genome is some 17 times greater than T2, and, for this reason, we chose the 17:1 (w/w) ratio for these mixtures (Table 3). In this manner, a new "genome" can be constructed which should be nearly 6% larger than the *E. coli* genome.

The data in Table 2 show an average $C_0t_{.5}$ of 5.3 for *E. coli* DNA and 0.38 for T2 DNA when renatured at an intermediate optimal temperature for both DNA species (61 C in SSC). The expected additivity for renaturation in the mixture was 5.7. This compares with an observed value of 5.6. Within the limitations of the

TABLE 2. *Renaturation kinetics of DNA mixtures with no sequence homology*

Source of DNA	Observed $C_0t_{.5}$[c]	Expected additivity $C_0t_{.5}$
Escherichia coli alone[a]	5.3	
T2 alone[a]	0.38	
Mixture		
E. coli + T2 (17:1)[a]	5.6	5.7
E. coli alone[b]	4.9 ± 0.1 (7)	
Bdellovibrio bacteriovorus 109 alone[b]	3.0 ± 0.2 (3)	
Mixture[b]		
E. coli + *B. bacteriovorus* (4.9:3.1)	7.7 ± 0.3 (3)	7.9

[a] Renatured at 61 C.
[b] Renatured at 65 C.
[c] Averages of two or more determinations.

standard deviation of our determinations (about 5% on individual DNA species) and the pipetting manipulations used to prepare each individual mix, the data indicated additivity of $C_0t_{.5}$ values and thus completely independent renaturation of *E. coli* and T2 DNA molecules.

The *E. coli* and *Bdellovibrio* mixture was prepared to give a "genome" approximately 60% larger than *E. coli* alone. When renatured under optimal temperature conditions, the $C_0t_{.5}$ was 4.9 for *E. coli* and 3.0 for *Bdellovibrio*. The equivalent genome mixture resulted in a $C_0t_{.5}$ of 7.7. Again, a value essentially identical to that predicted from the independently determined complexities was obtained.

An obvious extension of these studies would involve the preparation of DNA mixtures that are expected or known to have DNA polynucleotide sequences in common. Since the data from the above mixtures indicate that unrelated DNA molecules exhibit no apparent nonspecific aggregations or interactions to inhibit detectably their independent renaturation, the optical renaturation procedure might provide another method of demonstrating polynucleotide homologies. This can be demonstrated when renaturing mixtures exhibit cooperative renaturation kinetics, i.e., the $C_0t_{.5}$ of the mixture is less than additive. Results designed to demonstrate the feasibility of such an approach are shown in Table 3.

The first series of experiments was designed to demonstrate the effect on the $C_0t_{.5}$ when DNA mixtures containing essentially identical sequences are renatured together. Three strains of *E. coli* were chosen for these experiments. When renatured alone, *E. coli* B, K-12, and 15 TAU gave identical $C_0t_{.5}$ values and therefore contained essentially the same genome size, as would have been expected. The mixtures prepared of

TABLE 3. *Renaturation kinetics of DNA mixtures with sequence homology*

Source of DNA	Observed $C_0t_{.5}$
E. coli B alone	4.9
E. coli K-12 alone	4.7
E. coli 15 TAU alone	4.8
Mixture	
E. coli B + *E. coli* K-12 (1:1)	4.9
E. coli B + *E. coli* 15 TAU (1:1)	5.1
Salmonella typhimurium alone	5.2
Shigella flexneri alone	5.0
Mixture	
S. typhimurium + *E. coli* B (1:1)	9.3
S. flexneri + *E. coli* B (1:1)	6.0

equal weight amounts of DNA of *E. coli* B plus K-12 or B plus 15 TAU exhibited essentially the same $C_0t_{.5}$ as each sample alone (Table 3). This complete lack of additivity in $C_0t_{.5}$ determinations indicates that nucleations between homologous DNA molecules are just as successful as those formed in the heterologous case (B with K-12 or B with 15 TAU). This complete lack of additivity can be translated to mean 100% association or 100% "homology" in terms of sequence similarity. Conversely, the observations presented for *E. coli* B plus T2 and *E. coli* B plus *Bdellovibrio* which exhibit complete additivity (Table 3) are indicative of no polynucleotide sequence similarities or 0% homology.

To examine further the precision and usefulness of this technique in demonstrating polynucleotide sequence relationships, we next determined $C_0t_{.5}$ values for *E. coli* B/*S. typhimurium* and *E. coli* B/*S. flexneri* DNA mixtures. The choice of these cultures was based on the previous thorough examination of their DNA sequence relationships by using a variety of techniques by Brenner and his colleagues (6).

The $C_0t_{.5}$ values of the independently renatured samples illustrate an essential identity in the genome complexity of these two enteric bacteria with that of *E. coli* (Table 3). In the mixture *E. coli* and *S. typhimurium*, the $C_0t_{.5}$ is slightly, but significantly, less than additive. This would indicate a small amount of sequence similarity. The results of the *E. coli* and *S. flexneri* mixture show little additivity and indicate a much greater similarity in their DNA sequences. By utilizing the data from the four types of sequence relationships discussed, it was possible to develop an equation which correlates the observed $C_0t_{.5}$ of a mixture with the more familiar terminology of percent association or "homology" (as a percentage). The equation is

$$\{1-[\text{obs. } C_0t_{.5}^{mix} + (C_0t_{.5}^{100} - C_0t_{.5}^{0})]/C_0t_{.5}^{100}\} \times 100 \quad 3$$

The term obs. $C_0t_{.5}^{mix}$ is the observed $C_0t_{.5}$ of a renatured mixture, $C_0t_{.5}^{100}$ is the $C_0t_{.5}$ expected if the two DNA molecules are identical in sequence (complete lack of additivity), and $C_0t_{.5}^{0}$ is the $C_0t_{.5}$ expected for no sequence similarity (complete additivity of the independently measured $C_0t_{.5}$ values).

Table 4 lists the homology values calculated from equation 3 and compares the data with the expected or reported values.

The nucleotide sequences among the three *E. coli* strains were expected to be identical, and the calculated association reactions approached this theoretical limit (95 to 98%). The extent of homology in the *E. coli*/*S. typhimurium* intergeneric reaction was shown to be greatly affected by the temperature of the reaction (6). Our calculated homology value of 16% agrees quite well with the extrapolated value corresponding to our T_m −25 C as determined by Brenner et al. (6), who used the DNA-agar, membrane filter, and hydroxyapatite techniques. The mixture of *E. coli*/*S. flexneri* DNA molecules demonstrated a high degree of interaction, and our value of 79% is in excellent agreement with the literature (6).

Effect of the state of chromosome replication on renaturation kinetics. It can be predicted that the state of DNA replication would have an effect on renaturation rates, since a replicated chromosome will have some regions present in at least twice the concentration of other (unique) sequences. Several DNA preparations from *E. coli* B were prepared from logarithmic- or stationary-phase cultures grown at 37 C in glucose salts or in a rich (Penassay) medium. The consequences of the chromosome replication state on the $C_0t_{.5}$ and renaturation plots are shown in Table 5 and Fig. 2.

TABLE 4. *Quantitative polynucleotide sequence similarities determined from optical renaturation*

DNA mixture	Homology (%)[a]	Expected or reported (%)
E. coli B/K-12	98	100
E. coli B/15 TAU	95	100
E. coli B/*S. typhimurium*	16	9-30[b]
E. coli B/*S. flexneri*	79	80[c]

[a] All optical renaturations at optimum temperature of T_m −25 C. Homology calculated from equation 3.

[b] Brenner et al. (5). The range results from two reannealing temperatures, T_m −29 (30% binding) and T_m −20 (9% binding).

[c] Brenner et al. (5). There is relatively little effect of temperature on hybridization in the range of T_m −20 to T_m −29 C.

Table 5 illustrates that the $C_0t_{.5}$ values for stationary-phase cultures are independent of the growth medium. However, the $C_0t_{.5}$ for log-phase *E. coli* B DNA shows a reduction in $C_0t_{.5}$ attributable to an increase in the overall renaturation rate. DNA from the glucose-grown culture exhibits a slight but reproducible reduction of about 6% in the $C_0t_{.5}$, whereas the DNA extracted from cells in the enriched medium shows a 15% reduction.

An interesting consequence of the DNA replication state is demonstrable in the renaturation plots (Fig. 2). The line represents the observed and theoretical second-order plot calculated from the equation of Britten and Kohne (7), and this is linear when plotted in the form used by Wetmur and Davidson (24). The $C_0t_{.5}$ of this theoretical plot is 4.9, which corresponds to the observed $C_0t_{.5}$ of stationary-phase DNA samples. The solid points show the recorded reaction for the log Penassay DNA sample, and the open circles are those of the log glucose data. Both the increased renaturation rates and their departures from the theoretical second-order kinetics are apparent.

TABLE 5. *Effect of medium and growth phase on the $C_0t_{.5}$ of E. coli B DNA*[a]

Medium	$C_0t_{.5}$ Log-phase DNA	$C_0t_{.5}$ Stationary-phase DNA
Glucose salts	4.55, 4.7, 4.55	4.8, 4.9
Penassay broth	4.2, 4.2	4.9 ± 0.1 (7)

[a] Cultures were grown to the appropriate growth phase on a gyrotory shaker at 37 C. Cells were treated with 5 × 10^{-3} M sodium azide and chilled before centrifugation.

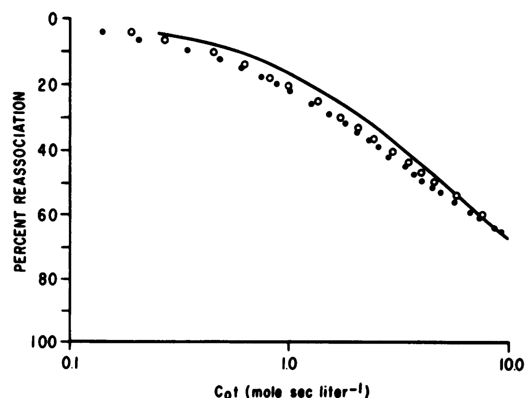

FIG. 2. *Optical renaturation of E. coli B DNA. DNA extracted from logarithmic growth in Penassay broth* (●), *mineral salts glucose broth* (○). *Theoretical second order plot for stationary-phase DNA. ($C_0t_{.5}$ = 4.9) calculated by the method of Britten and Kohne (7) is shown as a solid line.*

DISCUSSION

By employing four molecular-weight standards, it was possible to reveal the effect of the GC content on renaturation in our studies. When this phenomenon was quantitatively tabulated in the form of a relative rate constant versus GC base composition (Fig. 1), the slope could be superimposed on the tabulated data of Wetmur and Davidson (24). The calculated correction factor under the conditions of our determinations is approximately 1.8% of the observed $C_0t_{.5}$ for each mole % GC difference between the sample and *E. coli* DNA (the standard with 51% GC content).

The theoretical basis for the GC effect is not firmly established, but has been discussed (24). Renaturation of denatured DNA can be described in the terms of a reaction taking place by the formation (stable nucleation) of a number of "correct" base pairs followed by the sequential formation of adjacent base pairs. After successful nucleation, the reaction is essentially entirely in the direction of further base pairing. The rate of continual pairing under a predetermined series of conditions is at least partially controlled by an equilibrium constant for the formation of adenosine plus thymine (AT) or GC base pairs. In the derivation of a rate constant for nucleation, the equilibrium constant for GC pair formation is greater than that for AT pairs (24). From these considerations, this model predicts the observed parameters affecting renaturation. This includes the effect of temperature and the rate constant which is extrapolated from the GC effect. The magnitude and direction of the latter correction matches the data illustrated in Fig. 1.

There are some contradictions in the literature concerning the effect of GC base composition on renaturation kinetics. Bak et al. (1) showed that when *E. coli* DNA was used as a molecular-weight reference, a GC correction was necessary to establish the reported molecular weight for T4 DNA but not for *Haemophilus influenzae* DNA. It was conjectured that the effect of GC content on renaturation was peculiar to simple genomes such as those of phage. However, our data necessitate a correction for *Mycoplasma* DNA and would seem to rule out this supposition. However, Gillis, DeLey, and DeCleene (11) showed that, contrary to the data of Wetmur and Davidson (24), the rate constants decreased slightly with increasing GC content. This is also contrary to our data. The technique of DeLey and his colleagues involved measurements of initial renaturation rates only, and these were not second order. Since their procedure of measurement differs from ours, it is not possible to judge the significance of the difference in rate constants.

The optical measurement of DNA renaturation provides a means to determine polynucleotide sequence homologies in situations which may have proved impossible or difficult by isotopic techniques. For example, when it is not feasible to obtain high specific activity radioisotope-labeled nucleic acids, optical measurements would be the technique of choice. Furthermore, it is becoming increasingly apparent that there are technical limitations in the membrane filter technique for measuring DNA binding reactions, especially when a single label is used. During the course of incubation, variable amounts of DNA become eluted from the membrane (2, 22; *unpublished data*). The loss appears to depend on the temperature, time of incubation, and salt concentration and may amount to 15 to 90% loss of DNA. It is not known whether the loss is random or whether specific segments are lost preferentially. Binding assays may also exaggerate the amount associated which is not in full register. Such problems are not encountered with optical renaturation measurements.

Under carefully controlled conditions, the precision of the optical technique approaches that generally reported for other techniques (5 to 10%).

The major disadvantage of optical measurements is the limitation on sample number. Most spectrophotometers accommodate a blank and three samples, and an experiment may run as long as 6 to 8 hr. However, we feel that these disadvantages are counterbalanced by the uncertainties surrounding the membrane filter technique for measuring polynucleotide sequence similarities when a single isotopic label is used (8). It should be mentioned that additional information may be gathered from optical measurements of the physical characteristics of DNA. These, of course, include the determination of the GC content from the T_m (18) as well as the genome size from renaturation (7). These characteristics can be ascertained for several DNA samples in 1 day. The following day, the optical renaturation technique may be used to quantitate nucleotide sequence similarities among the samples.

Evidence favors a circular bacterial chromosomal structure which replicates from a fixed origin. When *E. coli* or *B. subtilis* is grown in glucose mineral salts with a generation time of about 60 min, the chromosome has a single replicating fork (12, 25). Such log-phase chromosomes in a nonsynchronous population are, on the average, 133% replicated. On a weight basis, stationary (aligned) chromosomes contain the greatest dilution of any DNA segment. This dilution is reflected in a 6% decrease in the $C_0t_{.5}$ of

the log glucose DNA sample. This observed value is close to the 7% value predicted on a theoretical basis (11).

When *B. subtilis* cells are grown in a rich medium (reduced generation time), the chromosome may replicate with three simultaneous growing points (26). In this situation, the redundancy of replicated segments is increased twofold over log glucose and fourfold over completely replicated chromosomes. As predicted, the greatest reduction in the $C_0t_{.5}$ (15%) was observed for renaturation of presumed three-forked *E. coli* B chromosomal DNA. These data collectively demonstrate the usefulness of renaturation measurements in detecting replication states of the bacterial chromosome. This technique might be applied to the possible confirmation of earlier work on chromosome replication in strains of *B. subtilis*. It was demonstrated by transformation marker frequencies that *B. subtilis* 168 does not accumulate completed chromosomes in the stationary phase as does strain W23 (26). Based on this evidence, DNA from stationary phase 168 should have a lower $C_0t_{.5}$ (faster renaturation) than DNA from stationary W23, provided there is little real difference in the respective lengths of each chromosome. We expect that synchronously replicating chromosomes would have the most drastic effect on renaturation. Synchronous replication might be measured during germination of *B. subtilis* spores, and the reduction in the $C_0t_{.5}$ could be compared to sequential changes in marker frequencies as replication proceeded.

ACKNOWLEDGMENTS

This investigation was supported by grant GB 8448 from the National Science Foundation. R.J.S. was the recipient of Public Health Service postdoctoral fellowship AI 36175 from the National Institute of Allergy and Infectious Diseases.

We thank William H. Kelton for his generous gifts of *Mycoplasma* lysates, and L. Daniel Inners, Julius Marmur, and Norman Davidson for their criticism and advice. Janet Bergendahl provided competent technical assistance.

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