APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1985, p. 1007–1013
0099-2240/85/101007-07$02.00/0

Vol. 50, No. 4

Determination of the Molecular Mass of Bacterial Genomic DNA and Plasmid Copy Number by High-Pressure Liquid Chromatography

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Received 1 April 1985/Accepted 11 July 1985

Relatively rapid methods for the determination of relative genome molecular mass (M_r) and the estimation of plasmid copy number have been developed. These methods are based on the ability of the Bio-Rad high-pressure liquid chromatography hydroxylapatite column to separate and quantify single-stranded DNA, double-stranded DNA, and plasmid DNA. Genome M_r values were calculated from reassociation kinetics of single-stranded DNA as measured with the hydroxylapatite column. Bacteriophage T4 DNA was used to establish a C_0t (moles of nucleotides times seconds per liter), or standard reassociation value. From this C_0t value, C_0t values for *Escherichia coli* B, *Beggiatoa alba* B18LD, and *Streptomyces coelicolor* were determined by comparative calculations. From those calculated C_0t values, the M_r values of 1.96×10^9 for *E. coli*, 2.02×10^9 for *B. alba*, and 3.28×10^9 for *S. coelicolor* were estimated. Plasmid concentration was determined from cleared lysates by comparing the integrated area under the phosphate buffer-eluted plasmid peak to values obtained with known amounts of plasmid. The plasmid copy number was estimated by multiplying the ratio between the amounts of plasmid and chromosomal DNA by the ratio between the M_r values of the chromosome and the plasmid. A copy number of 29 was obtained from a culture of *E. coli* HB101 harboring pBR322 grown to a culture density of 1.6×10^9 CFU · ml^{-1}.

Several methods have been used to determine the relative molecular masses (M_rs) of bacterial genomes. Autoradiography (9) and electron microscopy (32) were the first methods to be used. Due to the difficulties of these procedures other methods were sought. Chemical methods (42) are unsatisfactory because the number of nucleotides per cell and the state of replication of the chromosome are difficult to determine (31). A different approach to the determination of genome molecular masses was developed by Britten and Kohne (8), in which the renaturation of single-stranded DNA (ssDNA) to double-stranded DNA (dsDNA) was used. This process was shown to follow second-order reaction kinetics (8, 48). By using this approach, the M_r values of bacterial genomes have been calculated two ways. Britten and Kohne (8) calculated genome M_r from relative measurements of $C_0t_{1/2}$ (one-half of the reassociation as measured in moles of nucleotides times seconds per liter). Wetmur and Davidson (48) calculated genome molecular masses from the second-order reaction rate constant (k_2) and the M_r of the renaturing ssDNA.

Several methods have been used for the measurement of ssDNA reassociation, including: immobilization of DNA on agar (6) or nitrocellulose (17); digestion by ssDNA-specific nuclease (12); an optical method, based on the hyperchromicity of ssDNA over dsDNA (48); and separation of dsDNA from ssDNA by hydroxyapatite columns (28). Hydroxyapatite columns offer a variety of advantages in renaturation studies (28) and have also been used to isolate extrachromosomal DNA (11, 26, 43).

We have used a hydroxylapatite (HPHT) high-pressure liquid chromatography (HPLC) column for separation and quantitation of ssDNA, dsDNA, and plasmid DNA and have applied these methods to the determination of C_0t values for bacteria, the measurement of genome molecular masses, and the measurement of plasmid copy number. The advantages and disadvantages of this system are compared with established procedures.

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MATERIALS AND METHODS

Organisms and growth conditions. *Escherichia coli* B, a prototroph, was obtained from the culture collection in the Department of Microbiology at The Ohio State University, Columbus, Ohio. *E. coli* HB101 (*pro leu thr recA1 str lacY hsdM hsdR*) harboring pBR322 was obtained from J. S. Lampel. *E. coli* strains were grown with vigorous shaking at 35°C in L-broth culture medium (5) that was modified by the omission of glucose and the reduction of sodium chloride to 0.5% (wt/vol). Ampicillin (50 μg · ml^{-1}) and chloramphenicol (170 μg · ml^{-1}) were added when required. To obtain a high yield of plasmid DNA, plasmid pBR322 was amplified in the presence of chloramphenicol by the procedure described by Maniatis et al. (33).

Streptomyces coelicolor (OSU no. 686, from ISP no. 5049) was grown in yeast malt medium with vigorous aeration on a shaker at 30°C. Yeast malt medium contained (per liter): 10.0 g of glucose, 5.0 g of peptone, 4.0 g of yeast extract, and 2 ml of a trace elements solution. The trace elements solution contained (milligrams per liter): $ZnCl_2$, 40; $FeCl_3 \cdot 6H_2O$, 200; NiCl, 5; $CuCl_2 \cdot 2H_2O$, 2; $MnCl_2 \cdot 4H_2O$, 2; $Na_2B_4O_7 \cdot 10H_2O$, 2.

Beggiatoa alba B18LD has been described previously (34) and was grown at 30°C with rotary shaking at 150 rpm essentially as described by Strohl et al. (45). The medium contained (per liter): 500 mg of sodium acetate, 250 mg of NH_4Cl, 147 mg of $CaCl_2$, 10 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of K_2HPO_4, and 5 ml of a microelement solution (47).

Preparation of chromosomal DNA. For the isolation of

DNA from *E. coli* B, 2 liters of cells were harvested by centrifugation, washed once in 1/2 volume of TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]), and suspended in 60 ml of TE buffer containing 2 mg of lysozyme · ml^{-1}. The mixture was incubated for 15 min at 37°C with occasional shaking, after which 20% sodium dodecyl sulfate (SDS) was added to make a final concentration of 0.5%. Pronase (20 mg · ml^{-1}) was added to a final concentration of 1 mg · ml^{-1}. After incubation for 30 min at 37°C, the SDS concentration was adjusted to 1.0% by the addition of a 20% SDS solution. Sodium perchlorate (5 M) was added to a final concentration of 1 M. Protein was then removed by sequential extractions with equal volumes of the following: redistilled phenol saturated with 0.1 M Tris hydrochloride (pH 8.0), phenol-chloroform (1:1), and chloroform. Nucleic acids were precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of cold (−20°C) 95% ethanol. The nucleic acids were pelleted by centrifugation at 10,800 × *g* (9,500 rpm; model SS34; Ivan Sorvall, Inc.) for 10 min and dissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (33). The SSC concentration was adjusted to 1× by adding an appropriate volume of 20× SSC (33). Ribonuclease A (10 mg · ml^{-1} in 10 mM Tris hydrochloride–15 mM NaCl [pH 7.5], heated for 15 min at 100°C to inactivate deoxyribonuclease activity) was added to a final concentration of 50 μg · ml^{-1}. This mixture was incubated at 37°C for 30 min. The ribonuclease was removed by extraction with an equal volume of phenol-chloroform (1:1) and then an equal volume of chloroform. The DNA was precipitated with sodium acetate and cold ethanol as described previously, pelleted, and dissolved in 0.12 M sodium phosphate buffer (pH 6.8). *S. coelicolor* DNA was isolated as described by Chater et al. (10) and dissolved in 0.12 M sodium phosphate buffer (pH 6.8). For isolation of DNA from *B. alba* B18LD, a 2-liter culture of trichomes was grown and harvested as previously described (45). The trichome pellets were suspended in 45 ml of TE buffer, solid lysozyme was added to make a final concentration of 2 mg · ml^{-1}, and the mixture was incubated for 15 min at 37°C with occasional shaking. Trichomes were lysed by the addition of 1% SDS (final concentration) and the DNA was purified essentially as described for *E. coli*, with the addition of a CsCl purification step (33). The purity of all DNA preparations was estimated by the ratio of A_{260} to A_{280}. DNA with an A_{260}/A_{280} ratio of 1.8 to 2.0 was considered pure enough for further analysis.

Preparation of DNA fragments. The DNAs used in renaturation experiments were sheared to ca. 500 base pairs. The DNA fragment sizes were monitored by 3.2% acrylamide gel electrophoresis with bacteriophage lambda digested with *Bgl*II restriction endonuclease as the molecular mass standard. *E. coli* B DNA was fragmented by blending (5). All other DNAs were fragmented by sonication on ice for 5 min (10 30-s bursts) with a Branson Sonifier at a setting of 5. After fragmentation, the bacterial DNA solutions were filtered through a filter (pore size, 0.45 μm) and adjusted to concentrations of 250 to 350 μg · ml^{-1}, and the T4 DNA solution was adjusted to ca. 150 μg · ml^{-1} with sterile 0.12 M sodium phosphate buffer (pH 6.8). The concentration of DNA was determined spectrophotometrically at a wavelength of 260 nm as described by Maniatis et al. (33).

Preparation of purified plasmid DNA. After plasmid amplification, 1 liter of *E. coli* HB101 cells harboring pBR322 was harvested by centrifugation and lysed by the method of Holmes and Quigley (24). Plasmid DNA was purified by centrifugation in cesium chloride-ethidium bromide gradients (33). After extraction of the ethidium bromide with isopropanol, the plasmid solution was dialyzed against 0.12 M sodium phosphate buffer (pH 6.8). The purity of the plasmid was determined spectrophotometrically by using the ratio of absorbance at A_{260} and A_{280} (33).

Chromatography and reassociation kinetics. Fragmented chromosomal DNA (average size, 500 base pairs) in 0.12 M sodium phosphate buffer (pH 6.8) was denatured at 100°C for 10 min and then placed into a 60°C water bath (7) (time zero). For *S. coelicolor*, the samples were heated for at least 20 min at 105°C in a sealed tube. Samples (40 to 100 μl) were removed at time intervals (30 s to 12 h) and immediately frozen in an ethanol (95%)–dry ice bath.

Chromatography was performed at ambient temperature (ca. 21°C) on an Altex HPLC apparatus equipped with a Bio-Rad Bio-Gel HPHT column (100 by 7.8 mm) and guard column (50 by 4.0 mm). Rapidly thawed 20-μl samples were injected into a Rheodine HPLC sample injector equipped with a 20-μl loop. Elution was performed by using a step gradient from 0.1 to 0.3 M sodium phosphate (pH 6.8) at a flow rate of 0.45 ml · min^{-1}. It took 30 s for the concentration of the elution buffer to change linearly from 0.1 to 0.3 M. All buffers were filtered (pore size, 0.2 μm) and degassed. The buffers used for HPLC contained calcium and phosphate at the level of the solubility product for calcium phosphate (as described in the instructions accompanying the column) and were preserved with 0.05% sodium azide.

The ssDNA and dsDNA were monitored at 260 nm with a Hitachi spectrophotometer equipped with an Altex model 100-40 flow-through cell. Areas under the ssDNA and dsDNA peaks were integrated by a Hewlett-Packard 3390A integrator and calculated as percentage of total area. These were used to plot the fraction of DNA reassociated against log C_0t. C_0t is the product of DNA concentration in moles of nucleotides per liter, and the time (t) in seconds of reassociation. The rate of renaturation of fully denatured DNA is kinetically a second-order reaction (48). On the log C_0t plot, there is a nearly linear region on the curve that extends for about two logs (25). It was from this portion of the curve that a linear regression analysis was used to determine the C_0t value at 50% reassociation ($C_0t_{1/2}$). The genome size was estimated directly from the comparison of the $C_0t_{1/2}$ values with the standard C_0t value obtained for T4 bacteriophage (7).

Estimation of plasmid copy number. A 500-ml culture of *E. coli* HB101 harboring pBR322 was grown to a density of 1.6 × 10^9 cells · ml^{-1}. A cleared lysate was prepared from 60 ml of this culture by the method of Guerry et al. (21). The cleared lysate was made up of 6 M urea and 0.25 M sodium phosphate (pH 6.8). The urea-treated lysate was filtered (pore size, 0.45 μm), and a 200-μl portion was applied to the Bio-Gel HPHT column. The majority of the contaminating material (e.g., chromosomal DNA, RNA, and protein) was eluted at ambient temperature with a buffer of 0.25 M sodium phosphate with 6 M urea (pH 6.8) at a flow rate of 0.45 ml · min^{-1} (26). The plasmid was then eluted with a linear gradient of 0.01 to 0.4 M sodium phosphate.

A standard curve, correlating area under the plasmid peak to the amount of plasmid, was prepared by applying known amounts of purified plasmid onto the column and eluting with a linear gradient of 0.01 to 0.4 M sodium phosphate. The amount of plasmid in the experimental culture was determined with the standard curve, using the area under the plasmid peak. Total DNA was measured from a 200-ml portion of the culture by the diphenylamine reaction (16)

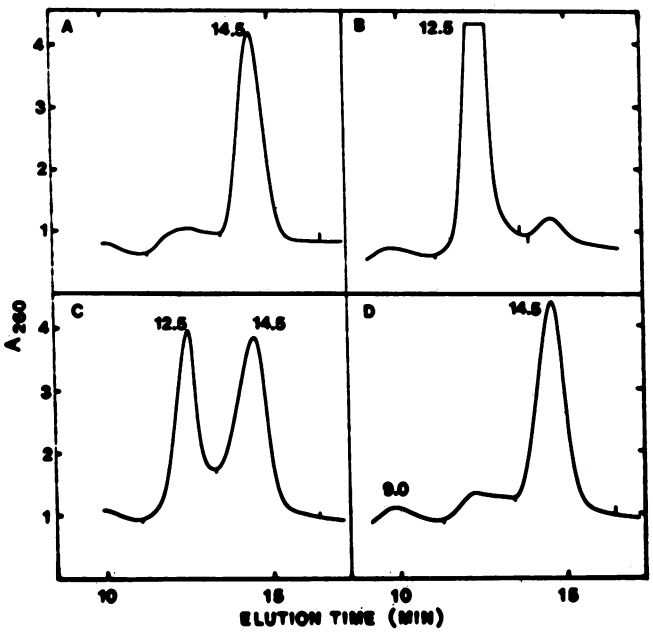

FIG. 1. Rapid separation of ssDNA from dsDNA with the Bio-Gel HPHT column. Samples (20 μl) containing 5.4 μg of renatured DNA were applied to the column. Elution was carried out by a step gradient formed by increasing the concentration of sodium phosphate from 0.1 to 0.3 M (pH 6.8) in 0.5 min (0.45 ml · min^{-1} at an ambient temperature). The buffer was maintained at 0.3 M for 4.5 min, during which time separation of the ssDNA and dsDNA occurred (retention time, between 12.5 and 14.5 min; there is ca. 12 min of delay time between injection and the chart recorder). The sodium phosphate concentration was then decreased from 0.3 to 0.1 M in 0.5 min to prepare for the next sample. Eluted peaks were detected by monitoring A_{260}. Low-molecular-mass DNA, ssDNA, and dsDNA eluted at approximately 9, 12.5, and 14.5 min, respectively. (A) Elution of dsDNA before heating to 100°C. (B) Elution of heated DNA that has been ca. 4% reannealed. (C) Elution of DNA that has been 62% reannealed. (D) Elution of DNA that has been 100% reassociated to dsDNA. Units of A_{260} are given as arbitrary values.

with salmon testes DNA as the standard. The copy number was calculated by multiplying the ratio between the amounts of plasmid and chromosomal DNA by the ratio between the molecular masses of the chromosome and plasmid (13).

Chemicals. All chemicals used were reagent grade or better. Bacteriophage T4 DNA, salmon testes DNA, ribonuclease A, lysozyme, chloramphenicol, ampicillin, and diphenylamine were purchased from Sigma Chemical Co., St. Louis, Mo. Bacteriophage lambda DNA was obtained from Bethesda Research Laboratories, Gaithersburg, Md., and *Bgl*II restriction endonuclease was obtained from International Biotechnologies Inc., New Haven, Conn. Ultrapure urea, necessary for the HPLC purification of plasmid DNA, was obtained from Schwartz-Mann, Cambridge, Mass., as catalog no. 821519.

RESULTS

Separation of ssDNA and dsDNA. The retention times on the HPLC hydroxylapatite column for ssDNA and dsDNA were approximately 12.5 min and 14.5 min, respectively (Fig. 1). A small peak with a retention time of approximately 9 min was also eluted. This peak represented DNA of low M_r and accounted for less than 5% of the total area under all three peaks (Fig. 1).

Relative molecular masses of genomes. The $C_0t_{1/2}$ values and relative molecular masses (M_r) of the strains tested are shown in Table 1. The $C_0t_{1/2}$ values obtained were 0.13, 2.30, 2.38, and 3.85 for bacteriophage T4, *E. coli*, *B. alba*, and *S. coelicolor*, respectively (Table 1). The correlation coefficients from the linear portions of the log C_0t plots ranged from 0.965 to 0.988 (Table 1). The $C_0t_{1/2}$ value obtained for T4 DNA was used as a reference for calculating the genome M_r values for the other test strains, based on the genome size of T4 as 166 kilobase pairs (50). Genome M_r was calculated directly from $C_0t_{1/2}$, as the two values are directly proportional over a factor of 10^9 (8). No corrections were made for the effect of base composition (3, 19, 48) or repeated sequences (8). The M_r values obtained were: *E. coli*, 1.96×10^9; *B. alba*, 2.02×10^9; and *S. coelicolor*, 3.28×10^9.

Figure 2 shows the log C_0t curves obtained with T4, *E. coli* B, and *S. coelicolor* DNA. Both *E. coli* B and T4 showed typical reassociation curves displayed with DNA which contains only unique or nonrepeated nucleotide sequences. The reassociation profile of *S. coelicolor* DNA contained a skewed profile at the lower C_0t values (Fig. 2).

Separation of plasmid DNA. Figure 3 shows the purification of pBR322 from a cleared lysate with the Bio-Gel HPHT column. The majority of contaminating material was removed in ca. 15 min during the urea elution, thus enabling the plasmid to be separated with a 0.01 to 0.4 M linear phosphate gradient. The plasmid was eluted approximately 13.3 min after the initiation of the phosphate gradient (Fig. 3).

Calculation of plasmid copy number. A standard curve correlating the integrated area under the plasmid peak to the amount of pure pBR322 plasmid is shown in Fig. 4. The correlation coefficient of this linear relationship was 0.997. The culture of *E. coli* HB101 harboring pBR322 contained

TABLE 1. Comparison of genome molecular masses of organisms tested

DNA source	G+C mol% of DNA	$C_0t_{1/2}$[a]	$C_0t_{1/2}$ relative to T4	Correlation coefficient[b]	Genome M_r	Genome size (base pairs)
T4	34[c]	0.13	1.00	0.980	1.10×10^8	1.66×10^5
E. coli	52[d]	2.30	17.8	0.965	1.96×10^9	2.94×10^6
B. alba	41 or 51[e]	2.38	18.4	0.982	2.02×10^9	3.03×10^6
S. coelicolor	74.7[c,d]	3.85	29.8	0.988	3.28×10^9	4.92×10^6

[a] Calculated from at least duplicate values from the nearly linear portion of the C_0t curves.
[b] Correlation coefficients were calculated from the linear portions of the log C_0t plots.
[c] The genome size for the T4 standard was obtained from Wood and Revel (50). The $C_0t_{1/2}$ value under standard conditions is 0.3 (50).
[d] The G+C mole percent values of the DNAs were obtained from Gzadek and Zakrzewska (20).
[e] The G+C mole percent has been calculated as 41 by T_m analysis (34) and 51 by bouyant density in cesium chloride (T. M. Schmidt and W. R. Strohl, unpublished data). There is no reason yet known for these differences.

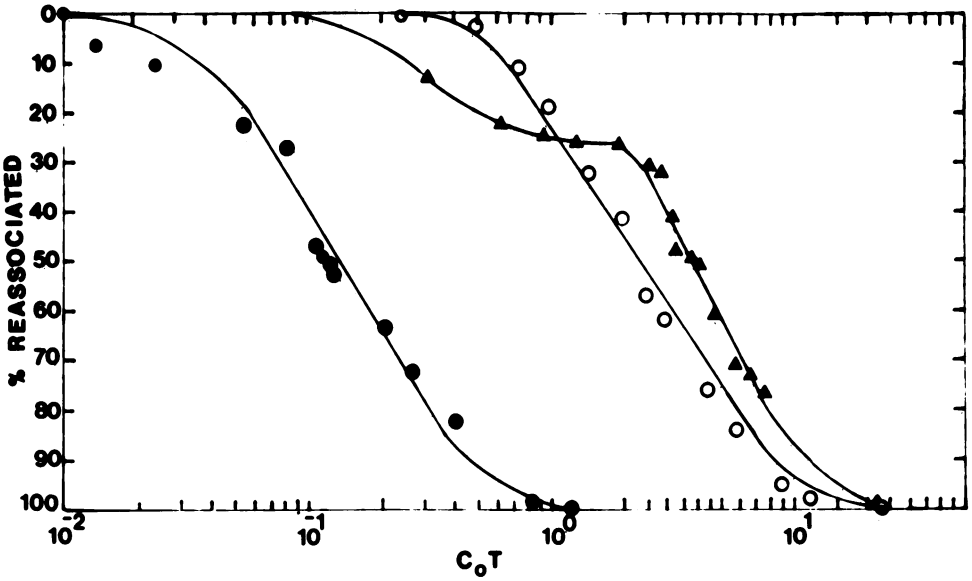

FIG. 2. Log C_0t plot of reassociation kinetics on the DNA from T4 (●), *E. coli* B (○), and *S. coelicolor* (▲). With T4 and *E. coli* DNA, each point represents at least the mean of duplicate determinations from a single experiment. Certain points were replicated in other experiments. With *S. coelicolor* DNA, each point represents the mean of duplicate determinations from two separate experiments.

0.37 μg of plasmid · ml^{-1}, as measured with the HPHT column by comparing the area (mean of duplicate determinations) under the plasmid peak to the standard curve. Total *E. coli* DNA, as measured by the diphenylamine reaction, was 10.62 μg · ml^{-1}. A copy number of 29 was then calculated for plasmid pBR322 as follows (13).

$$\frac{0.37\ \mu g/ml\ (pBR322\ DNA)}{10.62\ \mu g\ of\ total\ DNA\ per\ ml - 0.37\ \mu g\ of\ pBR322\ DNA\ per\ ml} \times \frac{2.35 \times 10^9\ (M_r\ of\ E.\ coli\ genome)}{2.878 \times 10^6\ (M_r\ of\ pBR322)} = 29\ (copy\ number\ of\ pBR322)$$

DISCUSSION

The methods developed and used in this research are based on the ability of hydroxyapatite to separate ssDNA, dsDNA (7), and plasmid DNA (11, 26, 43). This relatively standard procedure was expanded for use with HPLC by development of the HPLC HPHT column by Bio-Rad. Development of HPLC methods are advantageous because they are rapid, and combine qualitative separation with direct quantitation (via integration of peaks) of the separated products (e.g., ssDNA, dsDNA, plasmid DNA). Another HPLC technique for separation of ssDNA from dsDNA, in which a reverse phase column is used, has also recently been reported (30).

The C_0t value for T4 obtained using this HPLC procedure was 0.13, which is quite different from the 0.3 C_0t value for T4 obtained by different procedures (50). Thus with our procedure, M_r must be calculated by comparison of C_0t values with a standard DNA of known mass, such as T4. The relative M_r values of the *E. coli* B, *B. alba* B18LD, and *S. coelicolor* genomes were calculated by comparison with the T4 reference DNA of 166 kilobases (50). Our calculations for the M_r of the *E. coli* genome are about 27% lower than the value of 2.67×10^9 reported by Gillis et al. (19) and the value of 2.77×10^9 reported by Gładek and Zakrzewska (20). Both of these groups used the initial optical renaturation rate method (19). The M_r reported here for the *E. coli* B genome, however, more closely agrees with the value of 2.32×10^9 for *E. coli* JE5506, a K-12 derivative (23), reported by Yee and Inouye (51). Yee and Inouye obtained their measurement by quantitation of individual restriction fragments after two-dimensional agarose gel electrophoresis (51, 52).

Beggiatoas are sulfide-oxidizing, colorless, filamentous, gliding bacteria (29, 46, 47). The beggiatoas have been considered to be related to *Oscillatoria* spp., mostly because of their striking similarities in morphology and life cycle (29, 46, 47). The *B. alba* B18LD genome had aproximately the same mass as *E. coli* and was similar in mass to several *Oscillatoria* spp., which had M_r values of 2.50×10^9 to 4.38×10^9 (22). Recent data on the 5S ribosomal RNA of *B. alba* B18LD and other closely related beggiatoas indicate that they belong phylogenetically to group III of the purple photosynthetic bacteria (37). This would place the beggiatoas phylogenetically very distant from the cyanobacteria (37, 44); therefore, the similarities in genome masses are coincidental.

The streptomycetes, organisms that undergo a complex morphological differentiation, have been reported to have genome masses of ca. 1.5 times greater than that of *E. coli* (20). The M_r that we calculated for the *S. coelicolor* genome, 3.28×10^9, is ca. 13% smaller than the value of 3.75×10^9 reported by Gładek and Zakrzewska (20), who used the initial renaturation method of Gillis et al. (19). We consider this to be in reasonable agreement, especially considering the great differences in the techniques used. Benigni and his colleagues reported an M_r of 7.09×10^9 for the *S. coelicolor* genome (4), based on a spectrophotometric measurement of the reassociation constant (k_2; 48). There is some controversy, however, as to the accuracy of that method due to the potential error involved in the calculation of k_2 and the M_r

determination of DNA fragments by sedimentation (18). This might explain the abnormally high genome M_r values obtained for several organisms by that technique (18, 20).

The reassociation profile of *S. coelicolor* DNA suggests the presence of repeated sequences (2, 7). Examples of highly amplified (reiterated) DNA sequences have been reported in several members of the genus *Streptomyces* (15, 35, 36, 38–40). Repeated DNA sequences occur in other bacterial species (1, 27); however, very high levels of repeated sequences are unusual (15). *Caulobacter crescentus* has been reported to have enough repeat sequences to show up in a C_0t analysis (49). Moreover, Antonov et al. (2) have shown, by using C_0t data as well as other methods, that *S. coelicolor* contains about 5% reiterated DNA. Our data further indicate that reiterated sequences in streptomycetes may be measurable by using C_0t analyses. It is possible that our data may represent short segments of *S. coelicolor* that contain abnormally high local G + C mole percent levels that would not dissociate under the conditions we used. Antonov et al. (2), however, analyzed *S. coelicolor* for the presence of such sequences and found them to be lacking.

As mentioned previously, M_r values for the genomes were calculated on the basis of comparative $C_0t_{1/2}$ values. We did not account for the possible effect of base composition in our calculations. There is some controversy concerning the relationship between base composition and intrinsic renaturation rate. Gillis et al. (19) have described a reverse relationship between base composition and the intrinsic renaturation rate, while Wetmur and Davidson (48) and Seidler and Mandel (41) have reported a direct relationship.

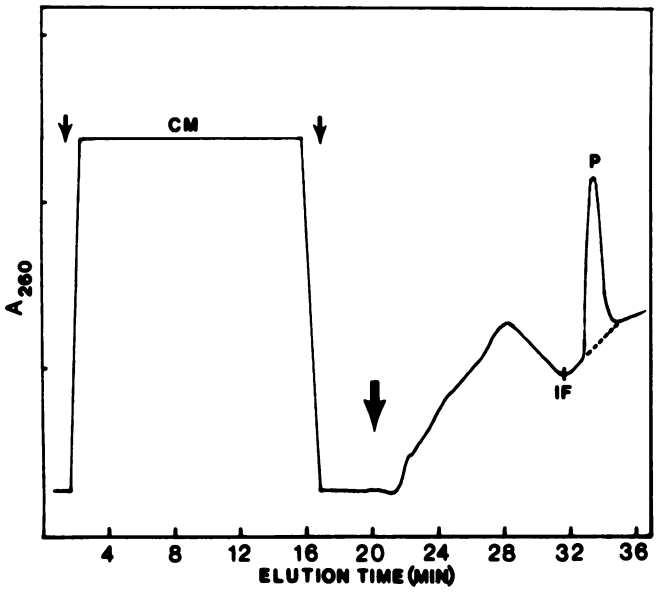

FIG. 3. Separation of plasmid with Bio-Gel HPHT column. Contaminating material (CM), e.g., chromosomal DNA, RNA, and protein, was eluted with 6 M urea–0.25 M sodium phosphate (pH 6.8) at a flow rate of 0.45 ml · min^{-1} (between small arrows). The column was equilibrated with 0.01 M sodium phosphate buffer (pH 6.8), and the plasmid was eluted by increasing the phosphate concentration from 0.01 to 0.4 M in 2 min at a flow rate of 0.5 ml · min^{-1} (started at large arrow). Plasmid DNA (P) was eluted ca. 13 min. after beginning the phosphate gradient. The integrator function (IF) was used so that only the area under the plasmid peak and above the background was integrated (see dotted line). A_{260} is given in arbitrary units.

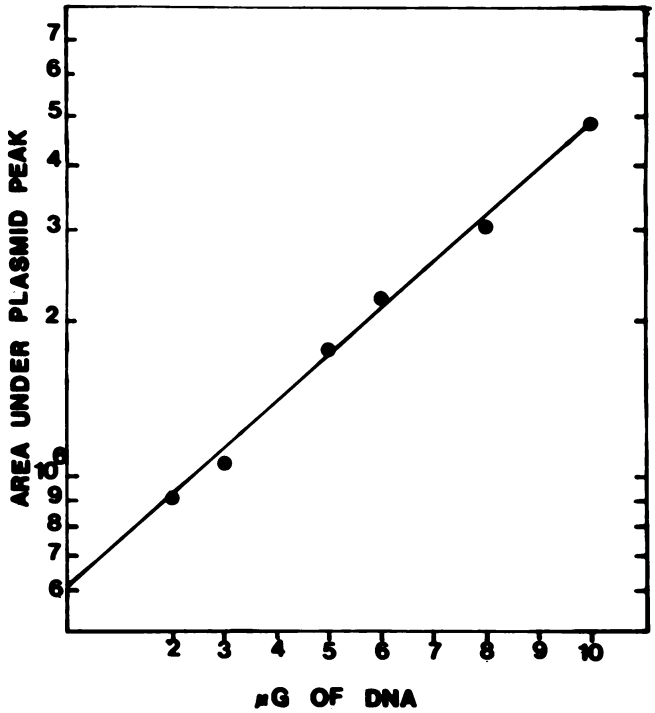

FIG. 4. Standard curve of pBR322 correlating the area under the plasmid peak to the amount of plasmid. The standard curve was generated by applying known amounts of purified plasmid to the Bio-Gel HPHT column. The plasmid was eluted as described in the legend to Fig. 3.

Bak et al. (3) have concluded that no relationship exists. Despite the controversy, the values reported here, with organisms which vary considerably in both genome size and base composition, agree closely with accepted values.

A copy number of 29 for pBR322 residing in *E. coli* HB101 agrees well with the accepted value of 30 (14). Methods for estimation of plasmid copy number have often been unreliable and inaccurate. In this report, we found that the areas under the plasmid peak from a test culture varied by ±15%. We do not expect significant losses from the crude lysate procedure that we used. Recoveries of extrachromosomal DNA of 95% (11) and 99% (43) by using hydroxyapatite have been reported. In addition, less than 0.5% of chromosomal DNA was present in the purified plasmid preparation (11). By the use of the standard curve, specific losses were probably eliminated. However, loss due to trapping of the plasmid in the chromosomal material in the preparation of the cleared lysate may have occurred.

This HPHT procedure described has potential advantages other than those described in this report. For example, it should be possible to combine HPHT separation of ssDNA and dsDNA with DNA-labeling procedures (e.g., nick translation with ^{32}P-deoxy-CTP) to develop rapid methods for quantitating the homology between DNA samples.

Due to the fragile nature of the packing, the Bio-Rad hydroxylapatite HPLC column would permit only about 200 to 400 separations. Moreover, the stability of the column was altered by the urea treatment used for plasmid separations. This may currenty limit the use of this technique for plasmid copy number due to the expense of the columns. Bio-Rad is, however, continuing its efforts to improve the stability and to lengthen the life of this packing (T. L.

Brooks, personal communication). Indeed, the last column which was supplied to us by Bio-Rad was able to tolerate significantly higher pressures than previous columns. We foresee that the columns will eventually be stable enough to run enough samples that even plasmid copy number determination will be a standard procedure. Moreover, alternative separation procedures, including temperature gradients, should be tried to maintain column stability during separation and quantitation of plasmids.

ACKNOWLEDGMENTS

This research was partially funded by a contract from Pioneer Hi-Bred, Inc. to W.R.S.

We thank Brian Burke for his assistance with the HPLC during the initial stages of this project. We also thank Tim L. Brooks at Bio-Rad, Inc. for his complete and sincere cooperation and for supplying us with additional HPHT columns.

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