

# Pulse-field electrophoresis indicates full-length mycoplasma chromosomes range widely in size

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## ABSTRACT

Full-size linear chromosomes were prepared from mycoplasmas by using gamma-irradiation to introduce one (on average) double-strand break in their circular chromosomes. Chromosome sizes were estimated by pulsed-field gel electrophoresis (PFGE) from the mobilities of these full-length molecules relative to DNA size references. Sizes estimated for *Ureaplasma urealyticum* T960 and 16 *Mycoplasma* species ranged from 684 kbp (*M. hominis*) to 1315 kbp (*M. iowae*). Using this sample, we found no correlation between the mobility of the full-size linear chromosomes and their G+C content. Sizes for *A. laidlawii* and *A. hippikon* were within the range expected from renaturation kinetics. PFGE size estimates are in good agreement with sizes determined by other methods, including electron microscopy, an ordered clone library, and summation of restriction fragments. Our estimates also agree with those from renaturation kinetics for both the largest and some of the smallest chromosomes, but in the intermediate size range, renaturation kinetics consistently provides lower values than PFGE or electron microscopy. Our PFGE estimates show that mycoplasma chromosomes span a continual range of sizes, with several intermediate values falling between the previously recognized large and small chromosome size clusters.

## INTRODUCTION

Mycoplasmas are low G+C content wall-less prokaryotes, grouped in the class *Mollicutes*, that are noted for containing the smallest chromosomes of any organism capable of independent replication and for being the smallest free-living cells. More than 100 mollicute species have been identified, and these occur widely as important pathogens or commensals in humans and other higher animals, in insects, and in plants [1,2]. Mollicutes have been shown to be evolutionary descendants of low G+C, gram-positive bacteria [3–7], and the small chromosomes of mollicutes are thought to result from losses of genetic material [3–8].

It is intriguing that mollicute chromosomes, rather than

occurring over a range of sizes, appear to fall into just two size classes, one composed of chromosomes clustering around 1600 kbp (ca. 1000 megadaltons) (*Acholeplasma*, *Spiroplasma*, *Anaeroplasma* and *Asteroplasma* species) and the second composed of chromosomes that cluster around 760 kbp (ca. 500 megadaltons) (*Mycoplasma* and *Ureaplasma* species) or approximately half the size of those in the first group [9–13]. Although chromosome sizes have been estimated for more than 30 mollicutes (representing nearly one-third of the cultivated species), no intermediate size mollicute chromosomes had been reported until recently [14]. Mollicute chromosome sizes have been estimated mainly by DNA thermal renaturation kinetics; for a few species, electron microscopy, analysis of an ordered clone library, or gel electrophoresis has been used to estimate size [9–18]. Concern has been expressed about the effect of the low G+C content of mollicute DNA on renaturation kinetic and pulsed-field gel electrophoretic determinations of chromosome size [1,19,20].

Here we report chromosome size estimates for 19 mollicute species determined from the mobility of their full-length chromosomes in pulsed-field gels. We also describe a new method for preparing chromosomes for pulsed-field gel electrophoresis (PFGE). This method employs gamma-irradiation to introduce double-strand breaks in circular chromosomes to yield a population of linear, full-size chromosomes that can enter a pulsed-field gel and form a sharp band. The procedure eliminates the need for restriction enzyme digestion and greatly reduces the manipulations and time required for preparing DNA for PFGE. In addition, the procedure should allow the isolation—free of host nucleic acids—of entire chromosomes from nonculturable bacteria such as mycoplasma-like organisms and rickettsia, and make them available for molecular genetic analysis.

## MATERIALS AND METHODS

### Bacterial strains

The majority of *Mycoplasma* and *Acholeplasma* strains used in this study are type strains and have been described previously [21]; *M. iowae*, and *A. hippikon* were kindly provided by J. Tully; *M. hominis*, *M. mustalae*, and *Ureaplasma urealyticum*

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were kindly provided by M. Barile, E.A. Freundt, and J. Robertson respectively. Organisms were grown in mycoplasma broth as described previously [21]. Mycoplasmas were grown until late log phase, chilled on ice, and harvested at 5°C.

#### Preparation of genomic DNA in agarose blocks

Cells were embedded in agarose blocks, lysed and deproteinized as described by Schwartz and Cantor [22]. Mycoplasmas usually were washed once in ice cold sterile TES (0.1 M Tris, 0.01 M Na<sub>2</sub>EDTA and 0.1 M NaCl, pH 8) and resuspended in the same buffer at a 30- to 100-fold concentration relative to the original culture. Cell suspensions were mixed promptly at 37°C with an equal volume of 1% low melting-low gelling agarose (InCert agarose, FMC, Rockland, ME) in TES, and cast into ca. 25 µl blocks (1.5×4×4 mm) in a lucite plastic mold and cooled. After solidification, the agarose blocks were expelled into warm SDS lysis solution (1% Na dodecylsulfate, 0.5 M Na<sub>2</sub>EDTA, pH 9.0) containing 1 mg Proteinase K/ml (4 to 5 blocks per ml) and incubated at 52°C for 18 to 48 h. Blocks were then prepared directly for electrophoresis or stored indefinitely at 4°C; to avoid SDS precipitation in the cold, the SDS lysis solution was replaced with warm sarcosine lysis solution (1% Na laurylsarcosinate, 0.5 M Na<sub>2</sub>EDTA, pH 9.0) and kept at 52°C for 15 min before refrigeration.

#### Cleavage of genomic DNA by irradiation

Genomic DNA agarose blocks were placed in sterile TE (10 mM Tris, 1 mM Na<sub>2</sub>EDTA, pH 8) in 2 ml polypropylene tubes (NUNC), cooled on ice and irradiated with a <sup>137</sup>Cs gamma-ray source (Mark I, Model 68, 22.5 kCi Dual Source Irradiator, J.L. Shepherd and Assoc., San Fernando, CA) at a dose rate of 67.7 Gy per minute (Gy = Gray = 100 rads; Fricke ferrous sulphate dosimetry, all doses within ± 5%; samples were rotated at ca. 12 rpm to provide a more uniform dose distribution). Irradiation times were usually 1.85 min (periods of 0.46, 0.92 and 1.85 min were used corresponding to gamma-ray doses of 31, 62 and 125 Gy).

Following irradiation, blocks were used for electrophoresis or stored for subsequent use. (Blocks may be stored refrigerated for a few weeks or returned to sarcosine lysis solution for long term storage at 4°C.) Blocks were rinsed two more times (10–15 minutes each) in a few ml of TE, drained on the tube wall and inserted into gel wells; wells were sealed with 1% agarose retained after pouring the gel.

#### Preparation of yeast chromosome and bacteriophage lambda DNA size markers

Chromosomes of *Saccharomyces cerevisiae* strain YPH 149 (a derivative of strain YNN 295 [23] with chromosome VII split at RAD2 [24] to yield markers of 1030 kb and 90 kb) were used as size markers. Yeast chromosomes were prepared by a modification (P. Hieter, unpublished) of the method of Schwartz and Cantor [22]. The sizes of the yeast markers were taken to be: 90, 210, 280, 350, 440, 550, 600, 680, 750, 790, 830, 940, 970, 1030, 1120 and 1600 kbp.

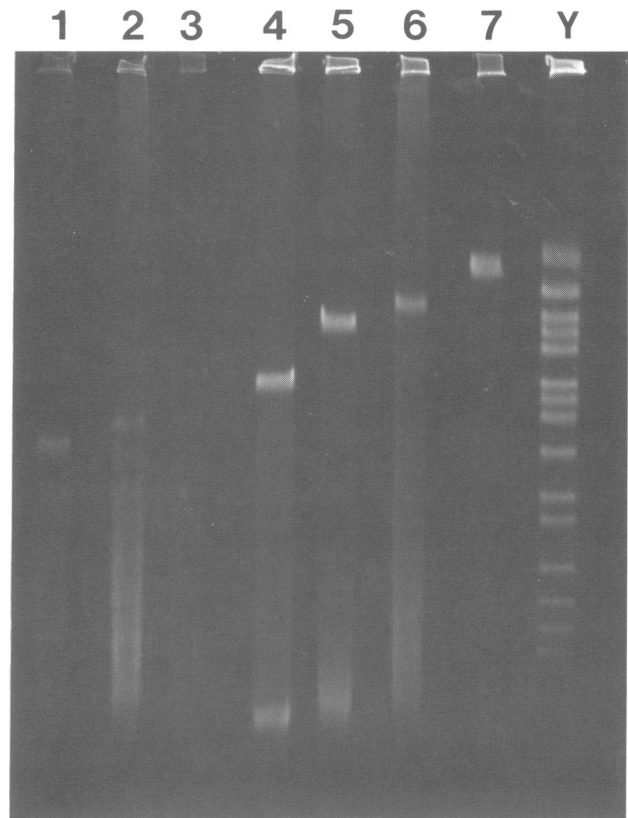
Concatamers of bacteriophage lambda (SAM 7) DNA used for size markers were prepared by modifications of a protocol provided by DNASTar, Inc; bacteriophages were purified through a glycerol step gradient, suspended in sterile 0.2 M Na<sub>2</sub>EDTA (pH 8.2), embedded in 0.5% agarose in TES and digested with 1 mg Proteinase K/ml in sarcosine lysis solution at 52°C for 48 h.

#### Pulsed-Field Gel Electrophoresis

Electrophoresis was carried out using a transverse alternating field gel-electrophoresis apparatus (GeneLine, Beckman Instruments, Fullerton, CA). Agarose gels (1%) were run in dilute TEA buffer (10 mM Tris, 0.5 mM EDTA (free acid) and 4 mM acetic acid, pH 8) maintained at 9 to 11°C. Gels were run for an initial 30 min period with 4 second switching intervals at 170 mA (300 V), followed by 60 second switching intervals at 150 mA for 18 h. This switching protocol produces good resolution where mobility appears to be a linear function of size as determined by the mobility of yeast chromosomes and lambda DNA markers. The *Acholeplasma* and *M. iowae* chromosomes were resolved by employing 135 sec switching intervals for 27 h under the same conditions. Gels were stained in ethidium bromide (1 µg/ml) for 30 min, destained in water for 10 min and photographed with panchromatic film under 300 nm UV illumination with a red 23A filter (Kodak). Photographic negatives (10×12.5 cm) were traced with an Ultrosan XL laser densitometer (LKB, Bromma, Sweden).

#### RESULTS AND DISCUSSION

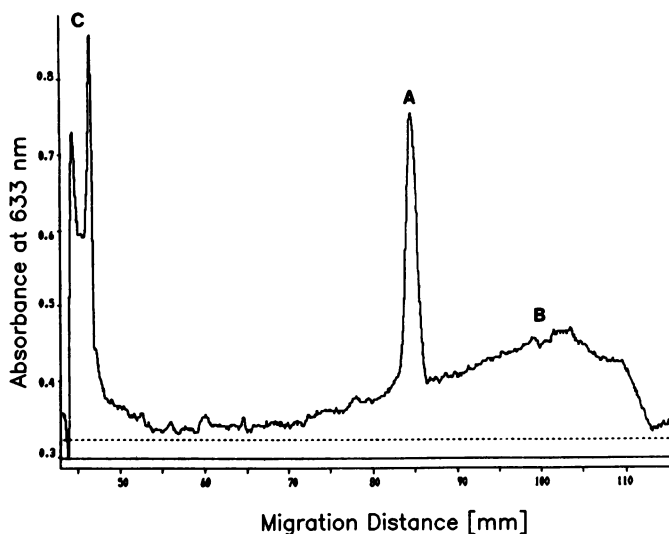
Figure 1 shows that gamma-irradiation of circular chromosomes produces DNA molecules that can enter pulsed-field



**Figure 1.** Pulsed-field gel electrophoresis of full-length *Mycoplasma* chromosomes made linear by gamma-irradiation. Lane 1, *M. hominis*; lane 2, *M. arginini*; lane 3, *M. orale*; lane 4, *M. pneumoniae*; lane 5, *M. pulmonis*; lane 6, *M. pullorum*; lane 7, *M. iowae*; lane Y, *Saccharomyces cerevisiae* strain YPH149 chromosome size standards. Chromosomal DNA was prepared in agarose blocks and irradiated as described in Materials and Methods. Electrophoresis was in a 1% agarose gel at 9–11°C with a current of 150 mA and switching intervals of 60 sec for 18 hr (preceded by an initial stage consisting of 170 mA with 4 sec switching intervals for 30 min) in a transverse field gel electrophoresis apparatus (GeneLine, Beckman Instruments).

electrophoresis gels and form a single sharp band. Unbroken bacterial chromosomes, which are usually circular, do not enter PF gels. Also, a smear, composed of random sized fragments produced by multiple breaks in chromosomes is visible in several lanes. We envisioned that chromosome sizes could be estimated by two independent methods. First, by comparing the mobility of the full-sized linear chromosomes to molecular weight standards, and second, by employing radiation target theory [25].

Several investigators have shown that ionizing radiation-induced breakage of DNA molecules can be described by the Poisson distribution [26,27]. Ostashevsky and Lange [28] have used this to show that the  $M_r$  of circular molecules can be determined from the induction of full-size linear molecules by the first double-strand break. Their approach provides two independent estimates of  $M_r$ : firstly by the hydrodynamic behavior of the full-size linear molecules (sedimentation rate, electrophoretic mobility, viscoelastic retardation time, etc.), and secondly, by the target theory, as follows. The dose which produces an average of one double-strand break (DSB) per molecule leaves 37% intact (in this case, as circular molecules remaining in the sample block), 37% with one DSB (chromosomes converted from circular to linear molecules, which enter the gel, and form a band of monodisperse size molecules), and the remainder having multiple DSBs and thus forming a random size distribution of DNA fragments (which appear as a smear in the gel). If the amounts of each of these 3 classes of DNA molecules could be measured accurately, it would be possible to estimate the size of the target chromosome by using the radiation dose that produces an average of 1 DSB per molecule and target theory [25,29]. The target theory based estimate gives  $M_r = 9.6 \times 10^9 / (D_{37\text{dsb}} \times G_{\text{dsb}})$ , where  $G_{\text{dsb}} = 0.1$  [30] and  $D_{37\text{dsb}}$  is the dose to produce an average of 1 DSB/molecule.



**Figure 2.** Densitometer trace of a photographic negative showing typical PFGE migration of gamma-irradiated DNA from a prokaryote (*M. hominis* PG21). A, trace of the band formed by full-length chromosomes which have been made linear by one double-strand break; B, a region composed of DNA fragments of various lengths produced by multiple double-strand breaks at random sites within chromosomes; C, Peaks produced by light scattering at the borders of the DNA sample block. Radiation dose: 125 Gy (12,500 rads). Electrophoresis was as described in Figure 1. Migration, from left to right, is measured from the lower edge of the sample block (C) to the center of the peak of the linearized chromosome (A).

The chromosome size estimates reported here are based on electrophoretic mobility comparisons. We were unable to use the target theory approach because light scattering at the borders of the sample insert produced spurious sharp high-intensity peaks on densitometric traces (Fig. 2). This prevented accurate integration of total DNA content in the gel. However, we hope to circumvent this problem by modifying the gel-sample interface.

Because gamma-irradiation produces just one sharp chromosome band in the gel, optimal pulse switching intervals for molecules of a given size range can be determined rapidly to allow comparison of chromosome mobilities to size standards. In contrast, restriction digest fragments can span a wide range of sizes and may require several trials to determine switching intervals suitable for measuring the mobilities of all fragments.

Occasionally we have observed in preparations from some species that unirradiated chromosomes were able to enter gels and form bands, presumably because endogenous nucleases or physical stress introduced one double-strand break in a sufficient number of chromosomes to produce a visible band. We have examined some conditions that might favor production of DS breaks, particularly altering the Proteinase K treatment, the availability of  $Mg^{2+}$ , the exposure to SDS or other detergents, and lysing the cells prior to mixing with agarose and pipeting into the mold. Although it is sometimes possible to obtain usable preparations in this manner, results are not predictable and preparations may be spoiled. Nevertheless, we are exploring these alternate methods of preparing samples for PFGE.

Figure 1 also shows that *Mycoplasma* chromosomes display a wide range of mobilities in pulsed-field gels. Mobilities of the 16 *Mycoplasma* species, *Ureaplasma urealyticum* T960, *A. laidlawii* and *A. hippikon* were determined similarly in replicate gels with yeast chromosome and lambda DNA markers (not shown). Apparent chromosome sizes determined from PFGE mobilities of full-length chromosomes from 19 mollicutes are listed in Table 1 together with values determined previously by other methods.

Chromosome sizes determined here for *M. hominis*, *M. arginini*, *M. orale*, *M. mustelae*, and *M. pneumoniae*, (Table 1) are concordant with earlier findings that mycoplasmas contain the smallest known chromosomes capable of independent replication. These PFGE size determinations for *M. hominis* and *M. orale* agree with sizes determined by DNA renaturation kinetics [11–13], and the size for *M. pneumoniae* agrees with that obtained from an ordered clone library [16]. These PFGE results affirm that these mycoplasmas contain exceedingly small chromosomes, however, the size estimates for *M. pneumoniae* from PFGE mobility and the clone library are approximately 15% larger than the renaturation kinetics value.

In contrast to these results, chromosomes from several other mycoplasmas and a ureaplasma exhibited lower mobilities. These mobilities indicate chromosome sizes ranging from approximately 900 kbp to more than 1300 kbp (Table 1). These sizes, though considerably smaller than those for most bacteria, are larger than the size range of 600 to 800 kbp determined for mycoplasmas and ureaplasmas by renaturation kinetics. Size estimates from electron micrograph contour length measurements are available for comparison for two of these species: for *M. capricolum*, the PFGE size is approximately 9% larger than the micrograph value; for *M. bovis*, two electron microscope values are available, 1038 kbp and 875 kbp [9]. The PFGE value is approximately 5% and 25% larger than the respective micrograph measurements, which in turn are about 55% and 30% larger than the DNA renaturation

TABLE 1. Apparent sizes of mollicute chromosomes

Organism	DNA % G+C <sup>a</sup>	PFGE	Chromosome size (kbp)		
			Renaturation kinetics	EM <sup>c</sup>	Other methods
<i>M. hominis</i> PG21	29.2	684	681 [11]		
<i>M. arginini</i>	27.6–28.6	735	606 [12]		
<i>M. orale</i>	27	732	712 [11]		
<i>M. pneumoniae</i> FH	39	840	727 [11]		850 [16]
<i>M. mustelae</i>	28.2	895			
<i>U. urealyticum</i> T960	28	910 (900) <sup>b</sup>	666; 712 [11]		
<i>M. pulmonis</i> PG34	27.5–29.2	950			
<i>M. bovirhinis</i>	24.5–27.3	955	666 [12]		
<i>M. edwardii</i> PG24	29.2	980			
<i>M. pullorum</i>	29	995			
<i>M. felis</i> CO	25.2	1055			
<i>M. galisepticum</i>	34–35	1070 (1050) <sup>b</sup>	742 [11]		
<i>M. neurolyticum</i> A	23	1075			
<i>M. bovis</i> Donetta	32.9	1080	666 [12]	1038; 875 [9] 1030 [10]	724 [18]
<i>M. capricolum</i> kid	25	1120			
<i>M. fermentans</i> GII	27.5–28.7	1160			
<i>M. iowae</i> 695	25	1315 (1280) <sup>b</sup>			
<i>A. hippikon</i>	n.d.	1540			
<i>A. laidlawii</i> A	32–36	1600	ca. 1660 [11]		1646 [18]

a. data from [2,21]; n.d., not done.

b. Sizes estimated by summing restriction fragments [14].

c. Sizes estimated from electron microscope contour length measurements.

value (Table 1). Another size estimate for *M. capricolum*, 724 kbp, has been obtained from a two-dimensional denaturing gel; this method appears promising but only one of the small chromosome *Mycoplasma* species and one *Acholeplasma* species have been examined using this technique [18].

Chromosome sizes previously determined by renaturation kinetics for *Acholeplasma* species were in the range of 1500 to 1700 kbp [13]. Chromosome sizes estimated here for *A. laidlawii* and *A. hippikon* were 1600 kbp and 1540 kbp respectively (Table 1), close to the sizes determined by renaturation kinetics.

Thus, several of the chromosome sizes estimated here agree with, or are within the range of sizes determined by, other methods; others do not agree; and for still others there is no basis for comparison, since they are determined here for the first time.

Existing chromosome size estimates for mycoplasmas and other bacteria have been determined mainly by DNA renaturation kinetics. Concern has been raised about renaturation kinetics determinations of mollicute chromosome sizes because the extremely low G+C content of these DNAs could be expected to affect renaturation [1,19]. The investigators who led in the early application of DNA renaturation kinetics to estimate mollicute chromosome sizes recognized the possible need for a correction factor for low G+C content; however, correction did not appear to be required since results for the most part agreed with measurements then available from other methods [11].

Chromosome size estimates from a recent PFGE study carried out on restriction digests from six mollicute species [14] also have been questioned [20]. These sizes, estimated from large fragments produced by restriction endonucleases that cut infrequently within these chromosomes, were all larger than expected from previous renaturation kinetic studies. It was suggested [20] that the size estimates for these chromosomes correlate with the low G+C content of their DNAs, and therefore low G+C *Mycoplasma* DNAs must exhibit anomalous behavior in PFGE, leading to slower mobilities and incorrect genome size values. Our results, based on a much larger sample, show that several different

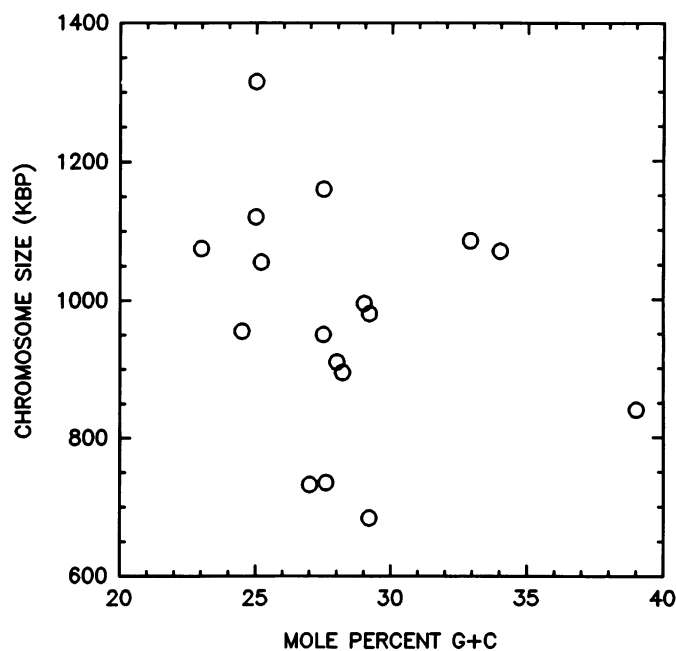


Figure 3. *Mycoplasma* and a *Ureaplasma* species chromosome sizes estimated from mobilities of full-size linear chromosomes in PFE gels plotted against mole percent G+C content.

*Mycoplasma* chromosomes share similar or even identical average G+C contents, yet differ considerably in electrophoretic mobility in PF gels. A plot of the *Mycoplasma* and *Ureaplasma* genome sizes determined here versus DNA G+C content (Fig. 3) demonstrates the absence of any correlation between chromosome size and average G+C content (Pearson product moment correlation  $r = -0.24$ ,  $N = 17$ , not significant).

This lack of correlation does not rule out the possibility that

local sequence composition could affect molecular conformation and hence electrophoretic mobility. However, since no correlation was found between size and G+C content, the PFGE-based size estimates of the six mollicute species [14] are not invalidated and the three of these that can be compared (Table 1) agree quite closely with our own estimates. The agreement between the estimates obtained by summing restriction fragments of various sizes [14] and our estimates from full-length chromosomes is significant and indicates that anomalous mobility is not an important factor in these measurements.

Comparison of the PFGE-based estimates with the renaturation kinetics-based estimates (see Table 1) show good agreement for both the largest (1600 kbp) and some of the smallest (680–730 kbp) chromosomes, but in the intermediate size range, renaturation kinetics consistently provides estimates lower than those from both PFGE and electron microscopy.

Additional support for the reliability of our size estimates for the intermediate-size chromosomes is that our PFGE size estimate of 1075 kbp for *Chlamydia trachomatis* L2 (Neimark, Hammerschlag and Lange; unpublished data), a prokaryote (ca. 44% G+C) from another class, agrees closely with sizes obtained by electron microscopic contour length measurements and sedimentation (1000 kbp) [31]. Recently, a PFGE-based size estimate of 1450 kbp for *C. trachomatis* L2 was obtained by summing the sizes of restriction fragments [32]; this is higher than the value we obtained for presumably the same strain. We note however that some of the sizes taken for their yeast chromosomes (*S. cerevisiae* strain 288C) are larger than those reported for other *S. cerevisiae* strains [23,24].

Mobility of linear DNA molecules in PF gels may not be dependent solely on size. Variation in field strength is known to alter mobilities in pulsed-field systems, however the use of lambda ladder and yeast markers that migrate closely with the unknown chromosomes makes this an unlikely source of error in the data reported here. As shown above, there is no correlation between DNA base composition and mobility. Theoretically however, mobility may be affected by other factors such as sequence dependent conformation, also mentioned above. Few examples of mobility anomalies have been identified in PFGE. One of the better documented cases involves the anomalous migration of *S. cerevisiae* chromosome 12 [22,33]; the reasons for its anomalous migration are not understood [22,34].

In conventional electrophoretic gels, anomalous mobility in natural and synthetic DNAs has been demonstrated to be due to intrinsic curvature of the DNA helix axis (see [35–38] and references therein). These curved DNA molecules, for a given size, have greatly reduced mobilities in polyacrylamide gels but essentially normal [37] mobilities in agarose [35,37]. The greatest anomaly occurs when the bend is located near the center of the molecule [39]. The spacial configuration of DNA and its ability to flex and twist is strikingly dependent on local sequences, particularly oligo(dA)-oligo(dT) tracts [36,38,40]. DNA methylation also may affect curvature [41]; various mycoplasmas are known to differ in the extent of their DNA methylation [42]. The full-length chromosomes examined here could contain one or more curved regions distributed at various sites along the DNA chain, depending on where the random DS break is introduced. Whether curvature in these long chain DNAs is stable and can have a net effect on mobility in agarose as examined here is unknown; however, yeast linear chromosomes and lambda DNA concatamers examined under identical conditions do not behave anomalously.

Either the mobilities observed here reflect the true sizes of these

chromosomes or some mobilities result from anomalous migration behavior. If the mobility of some full-length mollicute chromosomes is retarded it is significant, since this would demonstrate that anomalous mobility can occur in DNA molecules that are many hundreds of kilobases long. If such retardation occurs, then PFGE size estimates for other bacteria, including *E. coli* [43] and *Haemophilus influenzae* (39% G+C) [44,45], based on summation of restriction fragment sizes, may contain this artifact. The PFGE-based size estimates of approximately 1,900 kbp for *H. influenzae* [44,45] are larger than the value (1600 kbp) obtained by renaturation kinetics [11].

As discussed above (see also Table 1), several mollicute chromosome size estimates determined by renaturation kinetics are consistently lower than size estimates obtained by PFGE and/or electron microscopy. The possibility exists that repetitive sequences in mollicute genomes contribute to these discrepancies. Before the advent of renaturation kinetic estimates of genome size, there was no convenient way to estimate bacterial genome size and such sizes were available for only a very few species. Subsequently, renaturation kinetics has provided size estimates for many bacterial genomes (see for example [46,47]). Kinetic analysis of nucleic acid renaturation determines DNA complexity, and for haploid genomes that do not contain repeated sequences, DNA complexity and genome size are synonymous. Several investigators emphasized that for this identity to be valid, bacterial genomes must not contain repeated sequences (see for example [46,47]). Renaturation kinetic studies of most eukaryotic DNAs indicate the presence of repeated sequences. Recognition that many bacterial genomes also contain repeated sequences requires that the effect of such sequences on renaturation kinetics-based estimates of genome size be considered.

Some information regarding repeated sequences in mollicute chromosomes is available for a few species: *M. pneumoniae* [48–51], *M. hyorhinae*, *M. hypopneumoniae*, and *M. flocculare* [52], and three *Spiroplasma* species [53]. However, sizes obtained by both PFGE and renaturation kinetics are available only for *M. pneumoniae* and here the difference is only ca. 15% (see Table 1). Two different repetitive DNA elements, showing a high degree of homology, have been identified in the genome of *M. pneumoniae*; one element of 300 bp and another of 150 bp appear at least 10 times and 8 times, respectively [48]. The repeated elements are dispersed on the chromosome and, in three cases, are linked by a homologous DNA sequence of 400 bp [48]. Whether, or to what degree, such repeated sequences contribute to the observed discrepancies is unknown. The development of PFGE, combined with the rapid preparation procedure described here, also provides a convenient method for measuring chromosome sizes and allows comparison to values obtained by renaturation kinetics. When a sufficient number of genomes have been characterized as to their size, as well as the content, length and distribution of their repetitive sequences, there may be an opportunity to determine the contribution of repetitive sequences to renaturation kinetics.

Given the uncertainty of renaturation kinetics-based size estimates, the PFGE estimates may be the more reliable. Nonetheless, if the sizes of several mollicute chromosomes are over-estimated by PFGE and all *Mycoplasma* and *Ureaplasma* chromosomes examined fall within a range of 600–800 kbp, then the apparent gap between the large and small chromosome mollicutes would be upheld. The confirmation of a size gap between mollicutes would suggest that there are global structural features in these chromosomes, constraints, that have mitigated against the formation and maintenance of intermediate size

chromosomes during the evolutionary descent of mollicutes from their walled bacterial antecedents. It is unlikely that this gap would be filled-in by searching further among culturable mollicutes since the chromosomes from nearly 40 mollicute species now have been examined and it seems improbable that by chance, only mollicutes possessing large and small chromosomes have been selected for examination. This question becomes moot if the size estimates presented here are indeed correct.

Clearly, an independent measure of chromosome size is needed. Viscoelastometry promises to provide a reliable method for such size measurements. It is not expected that G+C content should contribute significantly to the measured retardation times.

Nevertheless, if the sizes estimated here are correct, as they appear to be, then there is a continual span of *Mycoplasma* chromosome sizes and no substantial gap exists between the large and small chromosome mollicutes. The demonstration of a range of large to small chromosome mycoplasmas implies that global chromosomal constraints had little or no effect on chromosome size during the evolutionary descent of mycoplasmas from their larger chromosome antecedents.

While this manuscript was in preparation, a report appeared in which chromosome sizes for two *Ureaplasma urealyticum* biotypes (14 strains), four *Mycoplasma* species and *A. laidlawii* were determined with PFGE by employing restriction enzymes to cut chromosomes once [54]. The reported values that can be compared (*U. urealyticum*, 890 kbp; *M. hominis*, 720 kbp; *A. laidlawii* A, 1580 kbp) are close to ours, and provide additional evidence that there is fairly good agreement between renaturation kinetics and PFGE determinations for the largest and the smallest chromosomes but in the intermediate size range, renaturation kinetics consistently produces lower estimates. Their results taken with ours further support our suggestion that, unless the PFGE data are inherently flawed, there is no size gap between large and small chromosome mollicutes.

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