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The Genomes of the Family *Rhizobiaceae*: Size, Stability, and Rarely Cutting Restriction Endonucleases†

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The lack of high-resolution genetic or physical maps for the family *Rhizobiaceae* limits our understanding of this agronomically important bacterial family. On the basis of statistical analyses of DNA sequences of the *Rhizobiaceae* and direct evaluation by pulsed-field agarose gel electrophoresis (PFE), five restriction endonucleases with AT-rich target sites were identified as the most rarely cutting: *Ase*I (5′-ATTAAT-3′), *Dra*I (5′-TTTAAA-3′), *Spe*I (5′-ACTAGT-3′), *Ssp*I (5′-AATAAT-3′), and *Xba*I (5′-TCTAGA-3′). We computed the sizes of the genomes of *Bradyrhizobium japonicum* USDA 424 and *Rhizobium meliloti* 1021 by adding the sizes of DNA fragments generated by *Spe*I digests. The genome sizes of *R. meliloti* 1021 and *B. japonicum* USDA 424 were 5,379 ± 282.5 kb and 6,195 ± 192.4 kb, respectively. We also compared the organization of the genomes of free-living and bacteroid forms of *B. japonicum*. No differences between the PFE-resolved genomic fingerprints of free-living and mature (35 days after inoculation) bacteroids of *B. japonicum* USDA 123 and USDA 122 were observed. Also, *B. japonicum* USDA 123 genomic fingerprints were unchanged after passage through nodules and after maintenance on a rich growth medium for 100 generations. We conclude that large-scale DNA rearrangements are not seen in mature bacteroids or during free-living growth on rich growth media under laboratory conditions.

Rhizobium meliloti is one of the most thoroughly studied members of the family *Rhizobiaceae* at the genetic level, but the genetic maps (reference 2 and references therein) are of low resolution. For *Bradyrhizobium japonicum*, there is no circular genetic map of the chromosome. Molecular maps exist for nodulation (*nod*) and nitrogen fixation (*nif* and *fix*) regions in most species of (brady)rhizobia, yet they typically span no more than 50 kb for any given region (reference 2 and references therein). Physical maps, or macrorestriction maps (37, 38), generated by using pulsed-field agarose gel electrophoresis (PFE) and rarely cutting restriction endonucleases (REs), would be useful to investigators studying *Rhizobiaceae* because they would greatly facilitate the mapping of new genes as well as furthering the study of novel regions of the mainly uncharacterized genomes of the *Rhizobiaceae*.

The genomes of *Rhizobiaceae* can be complex in structure compared with other well-known procaryotic organisms (reviewed in reference 25). In fast-growing rhizobia, for example, symbiotically important genes are located on large extrachromosomal replicons, called megaplasmids (reviewed in references 9 and 16). In some rhizobial species, such as *R. meliloti*, all known strains possess megaplasmids that are larger than the genomes of mycoplasmas and almost the size of the *Haemophilus influenzae* Rd genome (21). There is some dispute about the estimated size of the *R. meliloti* chromosome (5, 7, 15), and an unusually large estimate was reported for the *B. japonicum* genome (7). The structure of the rhizobial genome, as well as its possibly dynamic nature (25), could play a role in bacteroid development and function. It is possible that some aspects of bacteroid differentiation and development occur via DNA rearrangements. The developing bacteroid's genome has been an object of great interest and some dispute, and there is uncertainty about the amount of DNA in bacteroids relative to that in their free-living counterparts (reference 45 and references therein).

In this work, we present data on the molecular size of the *R. meliloti* and *B. japonicum* genomes. In addition, we have determined by PFE whether genome rearrangements have occurred during bacteroid development in *B. japonicum* or during free-living growth under laboratory conditions.

(Some of these results were presented at the 12th Annual North American Symbiotic Nitrogen Fixation Conference, Iowa State University, Ames, Iowa, July 1989.)

MATERIALS AND METHODS

Reagents, strains, and growth conditions. Unless otherwise noted, all chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.), all agarose was purchased from FMC (Rockland, Me.), and all REs were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.), New England BioLabs (Boston, Mass.), or Stratagene Cloning Systems (La Jolla, Calif.). *B. japonicum* USDA 110, USDA 123, USDA 138, USDA 122, USDA 127 and USDA 129 and their respective serotype-specific fluorescent antibodies were gifts from Renee Kosslak (Department of Genetics, Iowa State University). All other *B. japonicum* strains were gifts from Michael Sadowsky (Soil Science Department, University of Minnesota, St. Paul). *R. meliloti* 1021 and AK631, bacteriophage N3 (24), and *Rhizobium leguminosarum* biovar trifolii ANU 843 were gifts from Sharon Long (Department of Biological Sciences, Stanford University, Stanford, Calif.). The identities of *R. meliloti* and *B. japonicum* cultures used to make agarose plugs were confirmed either by lysis with specific phage (24) or by reaction with strain-specific fluorescent antibodies (35). *Agrobacterium rhizogenes* A4 was a gift from James H. Zhou (Department of Genetics, Iowa State University). *Saccharomyces cerevi-*

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siae 501 was a gift from Jennifer Detrick (Stratagene Cloning Systems). *B. japonicum* was grown in AG medium (34), and *R. meliloti*, *A. rhizogenes*, and *R. leguminosarum* biovar trifolii were grown in L broth (23) supplemented with the appropriate antibiotics. All *Rhizobiaceae* were grown at 28°C and maintained on agar slants.

Preparation and restriction digestion of DNA in agarose plugs and of molecular weight markers for PFE. Genomic DNA in agarose plugs, made from free-living forms of (brady)rhizobia, was prepared by using the pronase E treatment as described by Sobral and Atherly (41). To prepare bacteroid genomic DNA in agarose plugs, *B. japonicum* bacteroids were isolated, as previously described (32), from nodules of hydroponically grown (33) *Glycine max* cv. Williams. The plants were inoculated 2 weeks after germination with a suspension of *B. japonicum* in late log phase (10^9 CFU ml^{-1}). Bacteroids were isolated 35 days after inoculation (DAI). Large peribacteroid units were diluted 1:20 in Pett IV buffer (38) and embedded directly in agarose as described for free-living bacteria. The typical yield of large peribacteroid units from 5 g of 5-week-old nodules was sufficient to make approximately 200 agarose plugs, each containing 1 to 2 μg of intact genomic DNA. Because the large peribacteroid units do not contain cell walls, the *Escherichia coli* lysis solution step was omitted and the plugs were placed directly into ESPe.

Lambda concatemers and yeast chromosomes, used as molecular weight markers, were prepared as described by Sobral et al. (42). Restriction enzyme reactions were done as described previously (42). Processing and blotting of PFE gels were done as described by Sobral et al. (42), and Southern hybridizations were done according to standard techniques (23).

PFE equipment and conditions. PFE was done with one of the following: a Pulsaphor Plus system equipped with a hexagonal electrode kit (Pharmacia/LKB), an experimental long or a standard transverse alternating-field electrophoresis (TAFE) unit (Beckman Instruments, Palo Alto, Calif.), or an electrophoresis device (ED [36]). Field inversion gel electrophoresis (FIGE) was done in 16-cm-long SE600 or 32-cm-long SE620 vertical slab gel units (Hoefer Scientific Instruments, San Francisco, Calif.). Pulsing of the electric fields was controlled with a PC750 pulse controller (Hoefer Scientific Instruments), a minipulse controller (IBI, New Haven, Conn.), a TAFE controller (Beckman), a PPI-200 power inverter (MJ Research, Boston, Mass.), or a FIJI HV600 system (IBI). One to 1.2% agarose gels were cast and run in modified 1×, 0.5×, or 0.25× Tris-borate-EDTA (TBE) (TBEM [27]). Nominal voltage gradients were 6 to 13 V cm^{-1}. The gels and the running buffer were cooled to an equilibrium temperature of 10 to 15°C during electrophoresis.

Identification of rarely cutting REs. The nucleotide sequences, restriction maps, and mono-, di-, and trinucleotide frequencies of all sequenced genes and noncoding regions from various *Rhizobiaceae* were analyzed and downloaded from GenBank release 59.0 via BIONET. The sequences were spliced by species by using the Intelligenetics (Palo Alto, Calif.) SPLICE program available within the SEQ program. Splicing was done by uniting the individual sequences head to tail, with spaces between genes to exclude nucleotide combinations that might not be present in *Rhizobiaceae*. Statistical analyses of the spliced data bases were also done with the Intelligenetics sequence analysis programs.

Image analysis and scanning densitometry of PFE gels. Image analysis of the photographic negatives of ethidium bromide-stained PFE gels was done by a densitometrical process employing a Zeiss SEM-IPS image analysis system (Zeiss-Kontron). The negatives were placed on a Chromapro IBAS configuration light box and viewed with a Panasonic WV-CD50 video camera. The negatives were transilluminated by the light box. A program was written to measure and analyze the grey values of the individual DNA fragments on the negatives, one lane at a time. Grey values indicated the presence of multiplets.

Scanning densitometry of the photographic negatives was done on a Zeineh soft laser scanning densitometer, model SL-504-XL (Biomed Instruments, Inc., Fullerton, Calif.), with a tungsten light source set at 7.0 gain and 3.0 scan speed (in the high-speed range).

Calculation of sizes of restriction fragments on PFE gels. For *R. meliloti* 1021 and *B. japonicum* USDA 123, at least five independent size measurements were taken by using at least four different pulsing regimens to maximize the resolution of all sizes of restriction fragments. Migration distances were measured from photographic negatives of the ethidium bromide-stained PFE gels. The measurements were made on wall projections of the negatives or on photographic prints (20 by 25 cm). These measurements were then checked with measurements generated from scanning densitometry and image analysis. Molecular weight standards on PFE gels were plotted, and regression and other statistical analyses were done with Sigma-Plot 3.10 (Jandel Scientific, Corte Madera, Calif.).

RESULTS

Identification of rarely cutting REs. To determine genome size and to generate large DNA fragments for PFE studies, we first identified REs that cut the genome of *Rhizobiaceae* infrequently. We analyzed genomic sequences of *Rhizobiaceae* available on GenBank release 59.0. Because *Rhizobiaceae* are GC-rich bacteria, the probability of target sites was calculated for five REs that recognize AT-rich target sites (*Ase*I, *Dra*I, *Spe*I, *Ssp*I, and *Xba*I). Predictions were based on G+C molar ratios and on mono-, di-, tri-, and tetranucleotide frequencies used as generators for Markov chains (1, 28). Analysis with Markov chains generally underestimated the fragment size, probably because of sample bias, although the ranking of the most rarely cutting REs was accurate (results not shown). In practice, REs that produced the fewest fragments per genome were as follows: *Ase*I, *Spe*I, and *Xba*I for *A. rhizogenes* A4, *R. meliloti* 1021 and AK631, and *R. leguminosarum* bv. trifolii ANU 843; and *Ase*I, *Dra*I, and *Spe*I for *B. japonicum* (18 different strains and isolates tested [reference 43 and data not shown]). *Spe*I was the enzyme that cut most infrequently for all *Rhizobiaceae* studied (Table 1 and results not shown).

Stability of whole genomic fingerprints in *B. japonicum*. We compared the genomic fingerprints of three forms of *B. japonicum* USDA 123: bacteroids harvested at 35 DAI, isolates from crushed nodules, and free-living bacteria. The free-living bacteria were examined after approximately 20 generations and after more than 100 generations of growth on AG medium (Fig. 1 and data not shown). The identity of the strain was tested with cross-adsorbed fluorescent antibodies (35). The distribution of the five most rarely cutting RE sites in the bradyrhizobial genome was stable under the conditions used. Figure 2 shows genomic fingerprints of *B. japonicum* USDA 123 and USDA 122 and 35-DAI bacteroids of these strains. We did not observe any differences in the

TABLE 1. Sizes of *Spe*I-generated restriction fragments from *B. japonicum* USDA 424 and *R. meliloti* 1021

Fragment no.	Fragment size[a] (kb) ± SD	
	R. meliloti 1021	*B. japonicum* USDA 424
1	644 ± 33.0	467 ± 2.1
2	595 ± 36.7	401 ± 1.4
3	535 ± 33.8	383 ± 1.4
4	374 ± 7.8	345 ± 11.0
5	374 ± 7.8	289 ± 15.0
6	299 ± 4.7	268 ± 20.1
7	284 ± 1.2	258 ± 12.8
8	262 ± 6.7	258 ± 12.8
9	252 ± 7.0	239 ± 2.8
10	216 ± 13.3	228 ± 0.0
11	216 ± 13.3	218 ± 1.4
12	203 ± 18.0	208 ± 3.2
13	192 ± 17.1	203 ± 3.1
14	160 ± 13.2	193 ± 3.0
15	151 ± 13.0	176 ± 2.8
16	125 ± 12.6	164 ± 3.7
17	108 ± 8.2	164 ± 3.7
18	94 ± 4.6	153 ± 2.9
19	83 ± 5.8	144 ± 4.6
20	73 ± 9.7	144 ± 4.6
21	62 ± 9.2	129 ± 4.3
22	52 ± 4.4	121 ± 4.7
23	43 ± 1.4	114 ± 4.4
24		105 ± 6.2
25		96 ± 9.8
26		91 ± 8.2
27		84 ± 4.9
28		79 ± 5.6
29		75 ± 5.6
30		73 ± 0.0
31		68 ± 1.5
32		62 ± 2.8
33		55 ± 6.4
34		49 ± 8.5
35		46 ± 0.0
36		45 ± 7.1
Total	5,397 ± 282.5	6,195 ± 192.4

[a] Mean fragment size is 234.65 kb for *R. meliloti* 1021 and 172.08 kb for *B. japonicum* USDA 424.

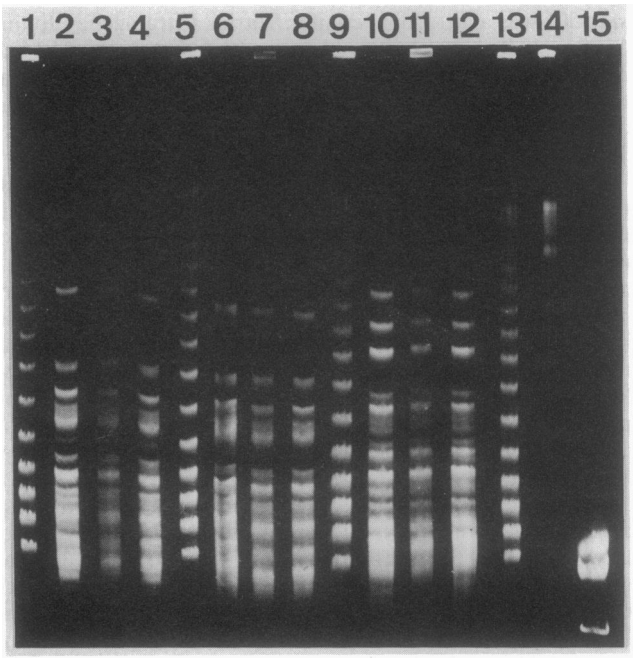

FIG. 1. Twenty-centimeter-long FIGE gel of *B. japonicum* USDA 123 genomic fingerprints. Lanes 1, 5, 9, and 13, Lambda concatemers; lanes 4, 8, and 12, plugs made from inoculum; lanes 3, 7, and 11, plugs made from isolates from crushed 35-DAI nodules; lanes 2, 6, and 10, plugs made from the same nodule isolates after 20 generations of growth on AG medium; lane 14, *S. cerevisiae* 334 chromosomes; lane 15, lambda-*Hin*dIII digest. The following restriction enzymes were used: lanes 2 to 4, *Asn*I; lanes 6 to 8, *Ase*I; and lanes 10 to 12, *Spe*I. The PFE conditions were 1.0% agarose (SeaKem LE, FMC) in 0.5× TBEM, a forward pulse of 0.3 to 30 s over 20 h, a ramp factor equal to 5.0 h^{-1}, and a voltage gradient equal to 7 V cm^{-1}. The reverse pulse was maintained at a 1:3 ratio with the forward pulse.

fingerprints produced with any of the rarely cutting REs in any of the pulsing regimens used (Fig. 2 and results not shown). The bradyrhizobial genome organization appears to be unchanged after bacteroid development. Complete digests were not always obtained with bacteroid DNA in agarose plugs, especially with *Dra*I (results not shown). In fact, *Xba*I was the only enzyme capable of consistently digesting bacteroid DNA in situ without some smearing being observed. Smearing was not reduced by the addition of 2 mM spermidine to potassium glutamate buffer (28) (results not shown).

Genome size in *R. meliloti* and *B. japonicum*. We used 20-cm-long TAFE gels or 35-cm-long FIGE gels for greater separation. For sizing of larger fragments, TAFE, FIGE, and ED gels were used. Figure 2 shows a representative TAFE gel of *B. japonicum* and *R. meliloti* DNA plugs digested with *Spe*I. *Spe*I was chosen for genome size calculations because it produced the smallest number of fragments of all the REs tested. There were a large number of fragments present in the 50- to 200-kb size range, especially for *B. japonicum* digests (Fig. 2 and Table 1). Scanning densitometry and image analysis were used to clarify the number of fragments generated by *Spe*I digestion of *B. japonicum* and *R. meliloti* DNA (results not shown). Putative *Spe*I doublet fragments from *R. meliloti*, which were identified by scanning densitometry or visual inspection, were excised from the PFE gel, digested with *Ase*I, and submitted to a second FIGE run to determine whether more than one fragment was present in the original gel slice. Fragments 10 and 11 are on different replicons, as are fragments 4 and 5, which were also shown to be doublets (15a). Size estimates determined with different PFE equipment were compared, and they agreed within less than 10% for any given fragment. Another report (43), as well as data generated during these studies (not shown), indicates that 18 other strains and isolates of *B. japonicum* have genome sizes that are within approximately 20% of the size of the strain USDA 424 genome.

DISCUSSION

We used PFE as a novel way of studying the complex genomes of two members of the *Rhizobiaceae*, *R. meliloti* 1021 and *B. japonicum* USDA 424. First, we present evidence that no major DNA rearrangements occur in free-living or 35-DAI bacteroids of *B. japonicum* in soybean nodules. We chose 35-DAI bacteroids because they are fully developed in the soybean-*Bradyrhizobium* interaction (14).

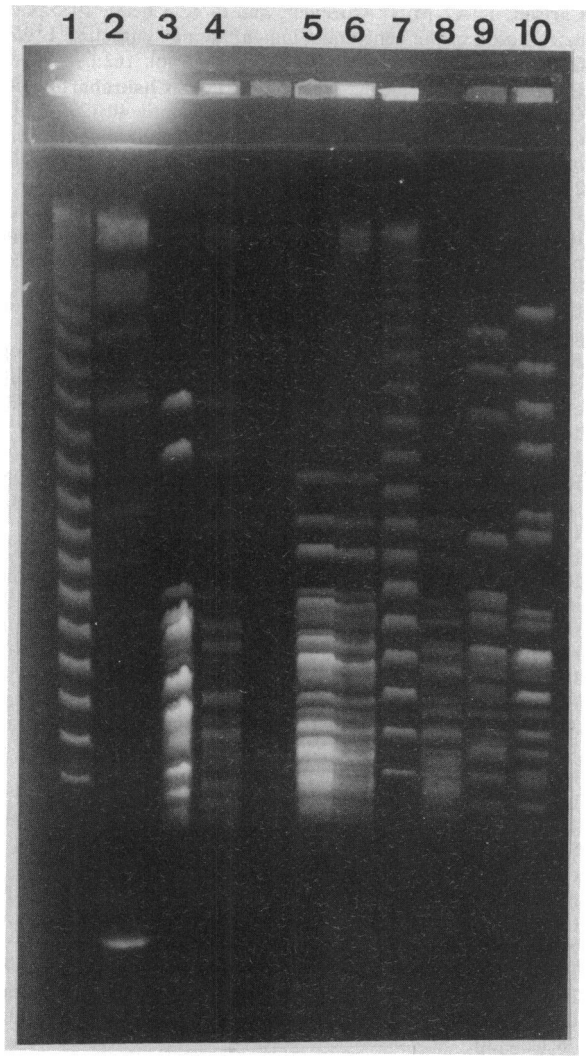

FIG. 2. Twenty-centimeter-long TAFE gel of genomic fingerprints of *B. japonicum* bacteroids and free-living bacteria and of *R. meliloti*, produced by digestion with *Spe*I. Lanes: 1 and 7, lambda concatemers; 2, *S. cerevisiae* 501; 3, free-living *B. japonicum* USDA 122; 4, USDA 122 35-DAI bacteroids; 5, free-living USDA 123; 6, USDA 123 35-DAI bacteroids; 8, free-living USDA 424; 9, *R. meliloti* 1021; 10, *R. meliloti* AK631. The PFE conditions were 1.2% FastLane agarose (FMC) in 0.5× TBEM, run at a constant current of 180 mA for 48 h. The initial pulse time was 3 s, and the ramp factor was 0.8 h^{-1}.

We are currently studying other stages of bacteroid development because it has been shown that younger bacteroids are less capable of dedifferentiating into vegetative cells (14, 29), suggesting that they may be more differentiated than mature bacteroids. At present, we cannot rule out the possibility of relatively small rearrangements occurring within large restriction fragments, and we cannot rule out the occurrence of simple inversions. Our present observations do rule out the occurrence of large-scale DNA rearrangements in mature bacteroids of *B. japonicum*. Genomic rearrangements are common in some *Rhizobiaceae*, notably in *R. leguminosarum* biovar phaseoli (13, 39, 40). Furthermore, repeated DNA sequences exist in *B. japonicum* (20), *Sinorhizobium fredii* (26, 31), and other *Rhizobiaceae* (12). These sequences could mediate rearrangements even during free-living growth; however, we saw no major rearrangements in *B. japonicum* during growth for up to 100 generations. It is possible that such rearrangements occur over longer periods of time in culture or that they are occurring at a very low rate and cannot be detected by PFE without selection. Despite our observation concerning the 35-DAI bacteroid genome, it is conceivable, though unlikely, that rearrangements occur early in the bacteroid development process and are reversed by 35 DAI; therefore, other time points will be sampled.

We calculate the *R. meliloti* genome size at 5.397 Mb (Table 1). Our calculated size agrees well with estimates based on reassociation kinetics (5.02 Mb [7, 15]), yet it still leaves questions unresolved. Electron microscopy (EM) has been used to measure contour lengths of *R. meliloti* megaplasmids (4, 5). These measurements estimated the sizes of the two megaplasmids present in strain 2011 (a sister strain of 1021) and of the megaplasmid of strain MVII/1 at approximately 1.5 Mb each. A recently constructed genetic map of pRmeSU47b estimated this *R. meliloti* megaplasmid to be 1.6 Mb (8). The chromosome of *R. meliloti* 2011 was determined by EM to be more than three times the size of the megaplasmids (5), which would suggest that it is at least 4.5 Mb in size. Data from EM measurements imply a considerably larger total genome size (at least 7.0 Mb) for *R. meliloti* 1021 than values obtained by reassociation kinetics (15) or from our results. By using PFE, we have not yet unambiguously resolved intact, undigested megaplasmids from *R. meliloti* 1021, so we cannot directly measure the size of each of the three replicons until the physical map has been completed. Preliminary results from preparative TAFE gels (15a) and *Pac*I (5′-TTAATTAA-3′) digests (40a) indicate that the *Spe*I genome size and the reassociation kinetics-based size estimates of the megaplasmids are correct. We must conclude, therefore, that the chromosome cannot be larger than the sum of the two megaplasmids. Our size for the *R. meliloti* chromosome (approximately 2.7 Mb) agrees with the estimates implied by reassociation kinetics studies (8, 15) if we accept the EM measurements (4, 5) as estimates for the sizes of the megaplasmids; however, our size does not agree with genetic data that compare the transfer frequencies of Tn*5* inserted into the chromosome and into the megaplasmids (17), nor does it agree with EM measurements of the chromosome of *R. meliloti* (5). Direct EM measurements and Tn*5* transfer frequencies agree with each other and imply that the *R. meliloti* chromosome alone is at least 4.0 to 4.5 Mb in size (5, 17). The genetic data assume a random insertion of Tn*5* throughout the *Rhizobium* genome, an assumption generally accepted as valid (8, 30). Yet if the *R. meliloti* 1021 chromosome is not much larger than the megaplasmids, as our results force us to conclude, why does it not migrate in the "in-well lysis" gels (16)? In fact, Hynes et al. (18, 19) reported observing the circular, covalently closed form of the *Agrobacterium tumefaciens* chromosome in such gels, suggesting that sometimes the chromosome can be resolved in these gels. It is reasonable to assume that the *A. tumefaciens* chromosome observed by Hynes et al. (18, 19) is at least 4 Mb in size (15). The resolution of the *A. tumefaciens* chromosome in in-well lysis gels suggests that the smaller *R. meliloti* 1021 chromosome should be seen in such gels. To our knowledge, however, no one has reported such an observation. We believe that these and other uncertainties about the structure and global organization of the (brady)rhizobial genome emphasize the need to construct physical maps for the most studied strains.

Many megaplasmids of the *Rhizobiaceae* are extremely

recalcitrant to curing (10, 16), suggesting that these replicons may harbor genes that are essential to the bacterium's survival. Supporting this idea is the fact that genes for thiamine biosynthesis (*thi*) and dicarboxylic acid transport (*dct*) have been localized to pRmeSU47b, a megaplasmid in *R. meliloti* SU47 (11). Our data show that approximately 50% of the genome is represented by the so-called megaplasmids if we use EM measurements for the sizes of the megaplasmids. We believe that this fact furthers the hypothesis that *R. meliloti* 1021 has three chromosomes, since it is rather difficult to accept the idea that more than 50% of the *R. meliloti* genome is nonessential. The localization to a replicon of essential genes, such as genes coding for rRNA and key biosynthetic genes, is considered sufficient evidence for the chromosomal nature of the replicon (44). To test definitively the three-chromosome hypothesis, we are determining whether essential genes can be localized to both megaplasmids. Alternatively, the isolation and the characterization of the origins of replication of these three replicons might yield more information about their nature.

The genome size of *B. japonicum* USDA 424 is approximately 6.2 Mb (Table 1). Our estimated genome size is approximately half that of previous estimates made by reassociation kinetics (7) or made as an unpublished observation (6). We have no explanation for this deviation. We do not believe, however, that USDA 424 is unique, since we have investigated 18 different *B. japonicum* strains and isolates (data not shown) and have not seen significant deviation from our calculated genome size for USDA 424. Lee and Smith (21), studying *H. influenzae*, showed that FIGE-derived genome size estimates were different from estimates derived by EM contour length measurement of the chromosome (22) or by determination of the amount of DNA per cell by colorimetric assays (3, 46). The estimates of the *H. influenzae* genome size produced by methods other than PFE varied by more than 100%.

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