INTRODUCTION

For a long time, it has been a paradigm that the bacterial chromosome consists of a single circular double-stranded DNA. This was based on genomic DNA organization in *Escherichia coli* and *Bacillus subtilis*. However, several bacteria have been found to have more than one chromosome; in addition, an unconventional linear structure has also been reported in several bacteria (for reviews, see Casjens, 1998; Volff & Altenbuchner, 2000). So far, we have only limited knowledge about the evolutionary and molecular mechanisms behind formation of linear or multiple chromosomes. Analysis of this mechanism might shed a new light on bacterial genomic structure as a whole. Because the *Rhizobiaceae* family contains species having various types of genome organization, their chromosomes are worth comparing with each other in order to determine the mechanism that led to creation of multiple chromosomes. Recent genomic information, including the complete sequences of several species in this family (Galibert *et al.*, 2001; Goodner *et al.*, 2001; Wood *et al.*, 2001), enables detailed comparison. However, there is still little genomic information about the biovar 2 group of *Agrobacterium*.

*Agrobacterium* species belong to the *Rhizobiaceae* family in the *Z* subgroup of *Proteobacteria*. Originally, bacteria belonging to the genus *Agrobacterium* were classified according to their phytopathogenicity, which is now known to be determined by large virulence plasmids (Herster & De Ley, 1984). This has caused taxonomic confusion. Therefore, *Agrobacterium* spp. have been divided into three distinct systematic units called biovar 1, biovar 2 and biovar 3 based on physiological characteristics. Later classification using rDNA sequences confirmed the biovar grouping as proposed by Sawada *et al.* (1993), although the conventional pathovar grouping is still in common use.

Genomic information has been collected intensively for biovar 1 group strains. Previous mapping of the *Agrobacterium tumefaciens* MAFF301001 strain (Suzuki *et al.*, 2001; De Costa *et al.*, 2001) as well as recent sequencing of the total genome of the C58 strain (Goodner *et al.*, 2001; Wood *et al.*, 2001) revealed that both linear and circular chromosomes possess essential housekeeping genes, rDNA sequences, and genes responsible for virulence. An *Agrobacterium* strain in the biovar 2 group has so far only been

Diverse types of genomic DNA organization have been found in *Rhizobiaceae*, especially among *Agrobacterium* species. Previous studies of *Agrobacterium* concentrated mainly on biovar 1 strains. Little attention has been given to biovar 2 strains. The biovar 2 genome consists of a large, circular chromosome and second megabase-sized replicon, as well as several plasmids. In this study two biovar 2 strains were analysed, *A. rhizogenes* (*A. radiobacter*) K84 and *A. rhizogenes* A4, by constructing physical maps of their chromosomes and mega-replicons. The maps revealed that in both strains their chromosomes consist of approximately 3-7 Mbp, while the mega-replicons are 2-6 Mbp circular DNAs. Gene mapping and comparative genomic analysis were performed based on the physical maps using Southern hybridization. It was found that rDNA, as well as analysed virulence and virulence-related genes, are present only on the chromosomes. The inter-chromosomal relationship between biovar 1 and biovar 2 strains was also analysed. Interestingly, there was a high similarity between the chromosomes of biovar 2 and the circular chromosomes of biovar 1, whereas similarity among the smaller megabase-sized replicons was restricted to each biovar. Based on these observations the possible relationship among large replicons in *Agrobacterium* biovars 1 and 2 is discussed.

**Physical and gene maps of *Agrobacterium* biovar 2 strains and their relationship to biovar 1 chromosomes**

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Diverse types of genomic DNA organization have been found in *Rhizobiaceae*, especially among *Agrobacterium* species. Previous studies of *Agrobacterium* concentrated mainly on biovar 1 strains. Little attention has been given to biovar 2 strains. The biovar 2 genome consists of a large, circular chromosome and second megabase-sized replicon, as well as several plasmids. In this study two biovar 2 strains were analysed, *A. rhizogenes* (*A. radiobacter*) K84 and *A. rhizogenes* A4, by constructing physical maps of their chromosomes and mega-replicons. The maps revealed that in both strains their chromosomes consist of approximately 3-7 Mbp, while the mega-replicons are 2-6 Mbp circular DNAs. Gene mapping and comparative genomic analysis were performed based on the physical maps using Southern hybridization. It was found that rDNA, as well as analysed virulence and virulence-related genes, are present only on the chromosomes. The inter-chromosomal relationship between biovar 1 and biovar 2 strains was also analysed. Interestingly, there was a high similarity between the chromosomes of biovar 2 and the circular chromosomes of biovar 1, whereas similarity among the smaller megabase-sized replicons was restricted to each biovar. Based on these observations the possible relationship among large replicons in *Agrobacterium* biovars 1 and 2 is discussed.
reported to have two circular mega-replicons of 4 Mbp and 2.7 Mbp, of which only one hybridizes with rDNA probes (Jumas-Bilak et al., 1998). So far, there are no data to confirm the chromosomal nature of the smaller mega-replicon despite its large size. That is why in this work we use the term mega-replicon, while the 3.7 Mbp replicon is called the chromosome.

In this paper we present what we believe to be the first physical and genetic maps of megabase-sized replicons among biovar 2 strains. Having constructed the maps, we were able to make interchromosomal comparisons between biovar 1 and biovar 2 strains. Chromosomes of two representative strains of biovar 1 were compared with mapped replicons of two biovar 2 strains. Based on the data, we discuss the relationship between chromosomes of different Agrobacterium biovars.

**METHODS**

**Bacterial strains and growth conditions.** Agrobacterium rhizogenes (A. radiobacter) K84 and A. rhizogenes A4 were grown at 28°C in YEB medium (1%, w/v, mannitol, 0.04% Difco Bacto-yeast extract, 0.02% NaCl, 0.05% K2HPO4, 0.02% MgSO4). A. tumefaciens MAFF301001 and A. rhizogenes MAFF301724 were grown at 28°C in LB medium.

**Genomic DNA preparation and restriction enzyme digestion.** Intact genomic DNA in agarose plugs was prepared as described elsewhere (Suzuki et al., 2001). Agarose segments containing genomic DNA were digested with PmeI, SwaI and PacI as previously reported (Suzuki et al., 2001), except that digestion with PacI was carried out using buffer recommended by the manufacturer.

**PFGE.** Electrophoresis was carried out using a contour-clamped homogeneous electric field (CHEF) electrode as previously described by Suzuki et al. (2001). Sizes of DNA fragments were calculated based on migration rate in PFGE, with Saccharomyces cerevisiae chromosomal DNA used as a molecular mass standard.

**DNA hybridization.** DNA fragments encoding MAFF301001 rDNA, chvA, chvB, chvD, chvE, chvG, chvl, acvB, glgP, pgm (exoC), miaA and ros were prepared as described elsewhere (Suzuki et al., 2001). Plasmid DNA from biovar 2 strains was extracted and purified as described by Suzuki et al. (2000). A random primer labelling method was employed to prepare isotopic probes using the DNA fragments as templates. Southern blotting and hybridization were carried out as described elsewhere (Suzuki et al., 2001), except final washings with 0.1 x SSC/0.1% (w/v) SDS were performed at 60°C instead of 65°C.

**RESULTS**

**Analysis of intact chromosomal DNA by PFGE**

A. rhizogenes strains K84 and A4 belong to the biovar 2 group, while A. tumefaciens MAFF301001 and A. rhizogenes MAFF301724 belong to biovar 1 (Table 1). Later in this paper they are referred to only by their strain names. Intact genomic DNA of these strains was subjected to PFGE. A single thick band at around 2 Mbp was observed for MAFF301001 and MAFF301724 (Fig. 1). These DNA bands are linear chromosomal DNAs of the two strains. No band was visible for K84 and A4, indicating that all of their replicons are circular and they remain intact without artificial linearization during sample preparation and electrophoresis.

**Table 1. Agrobacterium strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biovar</th>
<th>Size (kbp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. tumefaciens MAFF301001</td>
<td>1</td>
<td>2800 (circular) and 2000 (linear)*</td>
<td>Japanese Ministry of Agriculture</td>
</tr>
<tr>
<td>A. rhizogenes MAFF301724</td>
<td>1</td>
<td>3100 (circular) and 2000 (linear)†</td>
<td>Japanese Ministry of Agriculture</td>
</tr>
<tr>
<td>A. rhizogenes K84</td>
<td>2</td>
<td>3700 (circular) and 2600 (circular)†</td>
<td>N. McClure, Flinders University, Adelaide, Australia</td>
</tr>
<tr>
<td>A. rhizogenes A4</td>
<td>2</td>
<td>3700 (circular) and 2500 (circular)†</td>
<td>E. Nester, Washington University, Seattle, USA</td>
</tr>
</tbody>
</table>

* Suzuki et al. (2001).
† This study.

**Miscellaneous.** Autoradiographic detection was carried out using BAS-III imaging plates (Fuji Photo Film) with BAS2000 (Fuji Photo Film) and STORM (Amersham-Pharmacia) image analysers as recommended by the manufacturers. Other techniques were performed according to Suzuki et al. (2001) and Sambrook & Russell (2001).
Analysis of macro-restriction fragments by PFGE

For digestion of intact genomic DNA, two rare-cutting enzymes, PmeI and SwaI, were used. As shown in Table 2, the digestion patterns are polymorphic among the Agrobacterium strains. The digestions produced a small number of bands, 12 on average, which made the following mapping analysis easier. As summarized in Table 2, fragment size varied from 30 to 1600 kbp. No fragment smaller than 20 kbp was detected. It should be noted that some larger bands showed unusual migration patterns similar to the migration of the intact linear chromosome of biovar 1. Because of this, we used different pulse times (40, 70 and 120 s) and repeated the electrophoresis several times to correctly estimate fragment size and order of migration (data not shown).

The K84 Pme#1 band migrated in a different fashion from other bands due to its large size. It was especially visible in PFGE with a 40 s pulse time, when the Pme#1 band of K84 migrated faster than the Pme#4 band. Also, the band migrated faster than the Pme#2 and Pme#3 bands in electrophoresis run for a very long time (34 h) with a 70 s pulse time (data not shown). In the K84 strain, the Swa#7 band was clearly brighter than other bands of similar size, suggesting that it contains two fragments, which we named Swa#7a and Swa#7b.

The migration of the Swa#1 band of the A4 strain was also unusual due to the band’s large size. In digestions of A4 DNA, the Pme#1 band consisted of two fragments of similar size. They could only be distinguished by extraction of the band and subsequent digestion with SwaI. This digestion produced a banding pattern that could only be provided by two source fragments (data not shown). We arbitrarily named them Pme#1a and Pme#1b. Also, in the case of the A4 strain, three bands, Pme#11, Swa#7 and Swa#9, were relatively brighter than other bands of similar size. This suggests that each contains two fragments, which we named Pme#11a,b, Swa#7a,b and Swa#9a,b, respectively.

Physical map construction for the A4 strain

Individual macro-fragments were isolated from the bands in the PFGE gels and used as probes for Southern hybridization analysis to construct physical maps. Linkages between SwaI and PmeI fragments on each chromosome were revealed by Southern hybridization results; a representative result is shown in Fig. 2(a). The order of the Swa#6 and Swa#7b bands within the Pme#1b region was determined in experiments with Southern blots of

Table 2. Fragments produced by digestion of total genomic DNA of biovar 1 and 2 strains

Mean fragment sizes (kbp) were calculated using results of several digestions.

<table>
<thead>
<tr>
<th></th>
<th>A4</th>
<th></th>
<th>K84</th>
<th></th>
<th>MAFF301724</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pme</td>
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<td>1400</td>
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<td>#1*</td>
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<td>#1b</td>
<td>1200</td>
<td>#2*</td>
<td>1000</td>
<td>#2*</td>
<td>1050</td>
<td>#2*</td>
</tr>
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<tr>
<td>#3†</td>
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<td>640</td>
<td>#4†</td>
<td>670</td>
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<td>#6*</td>
<td>450</td>
<td>#6*</td>
<td>400</td>
<td>#6*</td>
</tr>
<tr>
<td>#6†</td>
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<td>#7a*</td>
<td>410</td>
<td>#7†</td>
<td>310</td>
<td>#7a†</td>
</tr>
<tr>
<td>#7†</td>
<td>260</td>
<td>#7b*</td>
<td>410</td>
<td>#8†</td>
<td>230</td>
<td>#7b†</td>
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<td>#8†</td>
<td>230</td>
<td>#9</td>
<td>220</td>
<td>#8*</td>
</tr>
<tr>
<td>#9†</td>
<td>175</td>
<td>#9a†</td>
<td>220</td>
<td>#10†</td>
<td>150</td>
<td>#9†</td>
</tr>
<tr>
<td>#10†</td>
<td>150</td>
<td>#9b‡</td>
<td>220</td>
<td>#11†</td>
<td>100</td>
<td>#10*</td>
</tr>
<tr>
<td>#11a†</td>
<td>75</td>
<td>#10*</td>
<td>50</td>
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<td>50</td>
<td>#11*</td>
</tr>
<tr>
<td>#11b‡</td>
<td>75</td>
<td>#11‡</td>
<td>30</td>
<td>#12*</td>
<td>65</td>
<td>#13*</td>
</tr>
</tbody>
</table>

*Biovar 2 chromosome fragments.
†Biovar 2 mega-replicon fragments.
‡Strain A4 plasmid fragments.
§Biovar 1 linear chromosome fragments.
||Fragments that possess rDNA sequences.
PacI-digested DNA and probes made of each of fragments Swa#1, Swa#6 and Swa#7 (data not shown). The resultant completed map revealed that the A4 chromosome was 3.7 Mbp in size and the second mega-replicon was 2.5 Mbp (Fig. 3, upper panel).

According to Nishiguchi et al. (1987), the A4 strain contains a hairy-root-inducing plasmid, pRiA4b. Southern hybridization with an isolated plasmid DNA fraction as a probe determined that four bands, Pme#9, Pme#11b, Swa#9b and Swa#11, come from the plasmid (Fig. 2a). These four fragments were not included in our chromosome map.

Physical map construction for the K84 strain
The same approach as described above was used for physical map construction of the K84 strain. As shown by the representative hybridization result in Fig. 2(b), we could detect overlap between Pmel and SwaI fragments, and subsequently determine the order of bands on the chromosomes. Using all the hybridization data, we were able to create a physical map for the K84 strain (Fig. 3, lower panel). The larger chromosome was 3.7 Mbp and the second mega-replicon was 2.6 Mbp. Unfortunately, the exact order of the short Pmel fragments within the Swa#7a,b region, i.e. the order of the Pme#7, Pme#9, Pme#10 and Pme#11 bands, could not be determined by any method used. The order of the Swa#10 and Swa#11 bands, i.e. which one is positioned next to the Swa#5 fragment, also could not be determined.

According to McClure et al. (1998), three plasmids are present in the K84 strain. We extracted and purified plasmid DNA and used it as a probe for Southern hybridization (data not shown). Strong signals were only visible in the sample well position. This indicates that the plasmids remained in the sample wells, even after digestion with the two restriction enzymes, because of absence of cutting sites for the enzymes, and did not interfere with physical mapping.

Mapping of rDNA and virulence genes
With the physical maps constructed for K84 and A4, we tried to localize a number of genes on the physical maps. rDNA sequences and fragments of 11 virulence and virulence-related genes from MAFF301001 were used as probes in hybridization experiments. Representative hybridizations are shown in Fig. 2. As shown in Fig. 3, all the localized genes were found on the chromosome in both K84 and A4. They were situated on one half of the chromosome, and the order of genes was the same in both strains.

Several of the 12 probes failed to detect specific genomic fragments of K84 and A4, or both, under the conditions applied. The failure was probably due to lower sequence homology, because the probes could hybridize with fragments of MAFF301001 in parallel experiments (data not shown). chvG and ros were not detected in A4, although they hybridized with K84 DNA. acvB and miaA sequences were not detected in either K84 or A4.
Chromosome similarity between *Agrobacterium* biovar 1 and biovar 2

After physical maps were constructed, we were able to compare chromosomes between *Agrobacterium* biovars, by interspecies and interchromosomal Southern hybridization experiments (Fig. 4). Chromosomes of two biovar 1 strains, MAFF301001 and MAFF301724, were compared with the megabase-sized replicons of the K84 and A4 strains.

The physical and genetic map of MAFF301001 was prepared earlier by Suzuki *et al.* (2001). For more reliable comparison between the two biovars, we also briefly characterized chromosomal DNA of another biovar 1 strain. In the MAFF301724 strain, digestion of isolated linear chromosomal DNA distinguished between linear-chromosome-derived fragments and circular-chromosome-derived fragments (data not shown). As a result, the linear and circular chromosomal DNAs in MAFF301724 were estimated to be 2 Mbp and 3·1 Mbp respectively (Table 2). rDNA was localized on both linear and circular chromosomal fragments in the strain (Table 2), as in other biovar 1 strains, C58 and MAFF301001.

As a biovar 1 circular chromosome probe, a mixture of Pme#1 and Pme#2 fragments of MAFF301001 was used. As shown in Fig. 4(a), none of the linear chromosomes bands of the MAFF301724 showed a signal with the probe, except for Pme#2 and Swa#4, which possess rDNA sequences. All the bands from the biovar 2 chromosomes hybridized, except for the shortest ones (Swa#10 in A4 strain, and Pme#12, Swa#6, Swa#12, Swa#13 in K84), while there was no hybridization with the smaller mega-replicon of biovar 2. These results indicate a high similarity between the larger chromosomes of biovars 1 and 2, and little similarity between the circular chromosomes of biovar 1 and the smaller mega-replicons of biovar 2.

To prepare a biovar 1 linear chromosomal probe, intact linear chromosomal DNA of the biovar 1 strain MAFF301001 was isolated from a PFGE gel (Fig. 1). The probe hybridized with all fragments derived from the linear chromosomal bands of MAFF301724 (except for the shortest, Swa#8 and Swa#9) (Fig. 4b). In biovar 2 strains, only bands that contain rDNA sequences showed weak signals. Based on this observation, we conclude that there is very low similarity between the linear chromosome of biovar 1 and both megabase-sized replicons of biovar 2.

Biovar 2 chromosomal DNA was represented by a mixture of Pme#1, Pme#2, Pme#3 and Pme#12 bands from the K84 strain. The band mixture was used for probe preparation. In MAFF301001, signals were detected on circular chromosomal
(a) Probe: MAFF301001 circular chromosome

(b) Probe: MAFF301001 linear chromosome

(c) Probe: K84 chromosome

(d) Probe: K84 2.7 Mbp mega-replicon
fragments Pme#2, Swa#1 and Swa#5, and on linear chromosomal fragments Pme#3 and Swa#3 (Fig. 4c). Both linear chromosomal fragments possess rDNA and chromosomal virulence genes. Curiously, there was no visible hybridization with the circular chromosome Pme#1 band, which does not possess rDNA loci and has fewer pathogenicity genes (Suzuki et al., 2001). In another biovar 1 strain, MAFF301724, signals were observed for circular chromosomal fragments Pme#1, Pme#3, Pme#4, Swa#1 and Swa#3, and for linear chromosomal fragments Pme#2 and Swa#4. Both linear chromosomal fragments were found to possess rDNA sequences (Table 2). All the fragments of the A4 strain chromosome hybridized with the probe. The data indicate similarity between substantial portions of the larger chromosomes between the two biovars, while similarity between the chromosome of biovar 2 and the linear chromosomes of biovar 1 is restricted to rDNA and its neighbouring sequences.

A mixture of Pme#4, Pme#5, Pme#6, Pme#9, Swa#7a,b and Swa#9 bands from the K84 strain was used to prepare a probe for the 2.7 Mbp mega-replicon of biovar 2. Under the conditions used, there was no visible hybridization with biovar 1 DNA (Fig. 4d). All bands derived from the smaller mega-replicon of strain A4 showed signals with the probe (except for the short fragments, Pme#10, Swa#8 and Swa#10). These results indicate that there is only very low similarity between the mega-replicons of biovar 2 and both biovar 1 chromosomes.

**DISCUSSION**

**Replicon size**

In this work, physical and genetic maps of two biovar 2 strains of *Agrobacterium* were constructed. As far as we know, these are the only maps available for biovar 2 chromosomes. Our results indicate that the circular chromosome of biovar 2 was approximately 3.7 Mbp in length, while its circular mega-replicon was approximately 2.6 Mbp. These sizes are smaller than those previously estimated by Jumas-Bilak et al. (1998). The total size of the two replications in biovar 2 was approximately 30% larger than that in biovar 1. The same size difference was observed between the larger chromosomes of biovar 1 and the chromosomes of biovar 2, as well as between the linear chromosome of biovar 1 and the mega-replicon of biovar 2.

**Location of genes**

Mapping of genetic markers in K84 and A4 strains revealed similarities, as well as some differences, in the location of important genes between biovars 1 and 2. This report, together with that of Jumas-Bilak et al. (1998), showed that rDNA sequences are only found on the chromosome in biovar 2 strains, whereas rDNA loci are located on both chromosomes in biovar 1 (Goodner et al., 2001; Wood et al., 2001; Suzuki et al., 2001).

We also found differences in the location of two chromosomal virulence genes. In the biovar 1 strain MAFF301001, the virulence-related genes pgm (exoC) and glgP in the glycogen metabolism operon were found on the linear replicon (Suzuki et al., 2001). Unlike in biovar 1, in the biovar 2 strains, pgm (exoC) and glgP were found on the chromosomes and not on the smaller mega-replicon. In this study, we could not find any genes located on the smaller mega-replicons of biovar 2.

With the data available, we cannot judge if the 2.6 Mbp replicon is a chromosome. This replicon should be analysed further, to learn what kind of genes it contains and if it is indispensable for the biovar 2 bacteria.

Other genes mapped to the larger chromosome of biovar 2 were generally positioned in the same order as those in the biovar 1 strain MAFF301001. chvG and chvI, together with an rDNA sequence, are located before the chvA and chvB genes, followed by chvD and chvE sequences located on the same restriction digestion fragment (Suzuki et al., 2001). Such organization was observed in both K84 and A4 strains (Fig. 3).

**Similarity of chromosomes**

The chromosomes of the two biovar 2 strains were highly similar to each other, and had considerable similarity with the circular chromosomes of biovar 1. However, it appears that the similarity level varies in different regions of the biovar 1 circular chromosome. It was restricted to approximately one half of the replicon, mainly to the regions harbouring rDNA sequences and chromosomal virulence genes. Similarity with the linear chromosome of biovar 1 was restricted to short fragments that also contain rDNA sequences.

**Origin of secondary chromosomes**

The mechanism that led to creation of secondary chromosomes remains unknown. Jumas-Bilak et al. (1998) suggested a process in which additional bacterial chromosomes could be formed through an intrachromosomal recombination event between tandemly duplicated regions of the genome. It might be similar to those used in the artificial dissection of a portion of the *B. subtilis* circular chromosome.
(Itaya & Tanaka, 1997) and subsequent formation of a new and stable replicon; all that was necessary for the excision event were two short repeat sequences, a plasmid origin of replication and housekeeping genes on the dissected section. Goodner et al. (2001) noticed that the biovar 1 linear chromosome possesses a region of high similarity with the Sinorhizobium meliloti chromosome, where such an excision event in a primordial chromosome of an agro-bacterial ancestor could have resulted in formation of the biovar 1 linear chromosome. Galibert et al. (2001) also proposed that large molecules could also be acquired from an external source and that both strategies could be possible within the same genome.

One interesting point is whether the secondary chromosomes were created before Agrobacterium spp. diverged into different biovars or whether they emerged independently. If Agrobacterium spp. diverged into biovars 1 and 2 after creation of a secondary chromosome in ancestral bacteria, then the similarity between the larger chromosomal molecules of both biovars and the similarity between the smaller chromosomal molecules should be comparable. However, although substantial portions of the larger chromosomes are similar between the biovars, we could not detect any similarity between the smaller chromosomes of biovars 1 and 2. This could be explained in two ways. Either the smaller chromosomes underwent larger and more frequent modifications to their gene contents in comparison with the number of changes on the larger chromosomes or, more probably, biovars 1 and 2 of Agrobacterium spp. acquired a secondary mega-replicon independently. If acquisition of the secondary chromosome was independent, it would mean that formation of a secondary chromosome was not a unique process in an ancestor of the agrobacteria, but rather was an important part of their evolution. However, a better understanding of the evolution of the secondary mega-replicon of biovar 2 will require further research, and experimental methods more precise than gross estimation by Southern hybridization.

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