Genome size and macrorestriction map of \textit{Xanthomonas campestris} pv. \textit{glycines} YR32 chromosome

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Abstract

\textit{Xanthomonas campestris} is an important plant pathogenic bacterium which causes severe diseases in a wide variety of plant species. We have generated a macrorestriction map of the \textit{X. campestris} (axonopodis) pv. \textit{glycines} chromosome employing pulsed-field gel electrophoresis (PFGE). Restriction endonucleases \textit{PacI} (5'-TTAATTAA), \textit{PmeI} (5'-GTTTAAAC) and \textit{SwaI} (5'-ATTTAAAT) digested the chromosomal DNA into three, five, and five fragments, respectively. In addition, intron-encoded restriction endonuclease \textit{I-CeuI} was employed to locate the position of the 23S rRNA genes (\textit{rrlA} and \textit{rrlB}). All of the generated restriction fragments were aligned along the chromosome using multiple restriction enzyme digestion and two-dimensional PFGE (2-D PFGE) in conjunction with Southern hybridization analysis. This physical map construction has revealed a single circular chromosome with a size of approximately 5 Mb. Two rRNA genes were localized on the chromosome map. Several genes involved in pathogenesis (\textit{xpsD}, \textit{opsX}, and \textit{pat}) as well as genes involved in the biosynthesis of xanthan gum (\textit{xanAB}, \textit{rfbCDAB}) were also localized.

Keywords: \textit{Xanthomonas}; Pulsed-field gel electrophoresis; Mapping; Circular chromosome

1. Introduction

\textit{Xanthomonas campestris} (axonopodis) pv. \textit{glycines} [28] is a plant pathogenic bacterium that causes bacterial pustules on soybean cultivars and is a member of the most diverse of the five species in the genus \textit{Xanthomonas} [2]. \textit{X. campestris} has become a model system for the study of pathogenesis because of its broad capacity for causing diseases in various species of plants. To date, our knowledge of the pathogenesis mechanisms of \textit{X. campestris} pathovars is rather limited. It is believed that extracellular proteases [27], lipopolysaccharides [11], exopolysaccharides [17], and endoglucanases [7] directly or indirectly play a role in the pathogenesis process. However, the mechanisms of how these enzymes interact to cause disease in plants are poorly understood.

Despite numerous studies of genes involved in pathogenesis [5,7–11,16,17,27] and extracellular polysaccharide biosynthesis [12], little is known about the structure and organization of the \textit{X. campestris}
2. Materials and methods

2.1. Bacterial strains and plasmid

Bacterial strains, plasmid and specific DNA fragments used in this study and their relevant characteristics are described in Table 1.

2.2. Growth conditions and media

Strains of Escherichia coli and Rhodobacter sphaeroides 2.4.1 were routinely grown in Luria-Bertani (LB) broth (yeast extract 5 g l\(^{-1}\), tryptone 10 g l\(^{-1}\), NaCl 10 g l\(^{-1}\)) pH 7.2 at 37°C [21], and X. campestris pv. glycines YR32 were routinely grown in LB+5% glucose pH 6.8 at 30°C. Plate cultures of X. campestris pv. glycines YR32 and the E. coli were grown on YDC (yeast extract 10 g l\(^{-1}\), dextrose 5 g l\(^{-1}\), CaCO\(_3\) 20 g l\(^{-1}\), Oxoid agar 15 g l\(^{-1}\)) [10] and LB with 15 g l\(^{-1}\) Oxoid agar, respectively. Whenever appropriate E. coli cultures were supplemented with ampicillin 100 μg ml\(^{-1}\), tetracycline 15 μg ml\(^{-1}\), chloramphenicol 34 μg ml\(^{-1}\), or kanamycin 50 μg ml\(^{-1}\).

2.3. Preparations of intact genomic DNA and restriction digests

Bacterial suspensions were prepared as follows: one loopful of a single colony was inoculated into 10 ml LB pH 7.2 (R. sphaeroides 2.4.1) or 10 ml LB+5% glucose pH 6.8 (X. campestris pv. glycines YR32), and incubated at 30°C for 24 h and 48 h, respectively. Bacterial suspensions were harvested by centrifugation and resuspended in PIV buffer (10 ml Tris-HCl, pH 7.5; 1 M NaCl) to a final concentration of approximately 2×10\(^8\) cells ml\(^{-1}\). Chloramphenicol (10 μg ml\(^{-1}\)) was added 15 min prior to harvest in order to synchronize chromosomal DNA replication.

The gel inserts (10×5×1 mm) were prepared as described by Smith and Cantor [24]. Restriction endonuclease digests were completed according to the protocol of Suwanto and Kaplan [25], with 8–15 U of enzyme for each digest, except for I-CeuI, for which we used 1 U of enzyme. Digestions with PacI or PmeI were performed at 4°C for 16 h, then at 37°C for 12 h, while digestions with SwaI were conducted at 4°C for 16 h, then at 25°C for 12 h (SwaI). Digestion with I-CeuI was performed at 37°C for 3 h. Multiple digestions were performed sequentially by washing the gel inserts with 1×TE buffer [21] for 1 h at 4°C, followed by incubation in the second or third restriction enzyme buffer for 15 min before subsequent digestion. All restriction buffers and conditions were carried out as recommended by the manufacturers (New England Biolabs or Boehringer Mannheim). SwaI was obtained from Boehringer-Mannheim Co. (Singapore). AseI, SphI, PacI, PmeI, and I-CeuI were obtained from New England (Singapore). Low melting point agarose for gel inserts was obtained from Bio-Rad (P.T. Diastika Biotekindo, Jakarta).

2.4. DNA fragment separation and macrorestriction fragment analysis

CHEF DR-II (Bio-Rad, Richmond, CA) [3] was used to separate DNA fragments. For separations of PacI, PmeI, and SwaI fragments, electrophoresis was carried out at 3.6 V cm\(^{-1}\) for 50 h with 50–650-s pulse times. For resolution of I-CeuI fragments, electrophoresis was conducted at 3.6 V cm\(^{-1}\) for 45 h with 50–650-s ramping pulse times. Two different pulse times of 20–25 s and 25–70 s were employed to separate AseI and SpeI small to medium, and large fragments, respectively. PFGE was conducted at 5.4 V cm\(^{-1}\) for 22–24 h. For all gels, we used 1% multi-purpose agarose (Boehringer-Mannheim) or ultra-pure agarose (Gibco BRL). Saccharomyces cerevisiae chromosomal DNA (New England Biolabs or Pharmacia) and SpeI-digested R. sphaeroides 2.4.1 genomic DNA [26] markers were used as molecular
size standards for the \textit{PacI}, \textit{PmeI}, \textit{SwaI}, and I-CeuI digests. For \textit{Asel} and \textit{SpeI} digests, lambda concatemeric DNA (New England Biolabs) and \textit{Asel}-digested \textit{R. sphaeroides} 2.4.1 genomic DNA [26] were used as a molecular size standards.

\textbf{2.5. Two-dimensional PFGE (2-D PFGE)}

Initial separation of single digestions of \textit{X. campestris pv. glycines} YR32 genomic DNA with either \textit{PacI}, \textit{PmeI}, or \textit{SwaI} was conducted as described in the DNA fragment separation, with the exception that 0.8\% multi-purpose agarose (Boehringer-Mannheim) was used instead of 1\% agarose. Each of the single digestions was performed in duplicate and run side by side in the same electrophoresis gel. Following separation, one lane of separated DNA fragments was cut out and stained with ethidium bromide (EtBr) for 15 min, and destained with sterilized water for another 30 min. The other lane consisting of the same DNA fragments was kept unstained. The DNA fragments in the stained gel were visualized using a UV transilluminator. The approximate location from the well of each DNA fragment was recorded. These data were then used to locate DNA fragments in the unstained gel. Each of the DNA fragments from the unstained gel was excised and washed by incubating it in 1.5 ml 1×TE buffer for 1 h at 37°C. This treatment decreased the concentration of boric acid and EDTA in the gel, since the concentration of EDTA in 1×TE buffer is lower than that in the 0.5×TBE running buffer. After three repeated washes, the gel slice was incubated with 150 µl restriction buffer for 1 h at 4°C. Incubation in the appropriate restriction buffer was also repeated three times. The restriction enzymes were added and digestions were performed as described previously [25]. The resulting DNA fragments were separated by PFGE using the conditions described in Section 2.4.

\textbf{2.6. Southern hybridization analysis}

Separated DNA fragments were transferred onto Photogene nylon membrane (Gibco BRL) using the capillary transfer method [21]. Because of the large size of many of the DNA fragments to be transferred, the DNA was first depurinated with 0.2 N HCl for 10 min, and washed in distilled water for 5 min prior to denaturation. Non-radioactive probes were prepared from specific DNA fragments derived from unique plasmids (Table 1) using the Bio-nick Translation kit (Gibco BRL). The probes were made by labelling each DNA fragment with biotin-14-dATP. Southern hybridization was performed at 42°C as described by Donohue et al. [6]. The washes were performed at 45°C, except for the rRNA probe at 50°C. For detection, we used the chemiluminescent assay procedure described by the manufacturer (Photogene detection system, Gibco BRL). The X-ray film used in this study was the product of Agfa (Germany).

\textbf{3. Results and discussion}

\textbf{3.1. Resolution of restriction fragments using rare cutting restriction endonucleases}

To construct a physical map of the \textit{X. campestris pv. glycines} genome, restriction endonucleases which cut its genomic DNA into a small number of fragments were sought. The mol\% G+C content of the DNA was generally used to narrow the range of restriction endonuclease to be tested [26]. Based on information that \textit{X. campestris} has 63–71 mol\% G+C DNA [2], we looked for enzymes with A+T-rich recognition sequences. Among the enzymes that we have tested, we found that \textit{PacI} (5’-TTAATTAA), \textit{PmeI} (5’-GTTTAAAC), and \textit{SwaI} (5’-ATTTAAAT) cut the DNA infrequently. \textit{PacI}, \textit{PmeI}, and \textit{SwaI} digested \textit{X. campestris pv. glycines} YR32 genomic DNA into three, five, and five fragments, respectively, with manageable distributions of DNA fragments and no overlapping fragments (Fig. 1). Double digestion of genomic DNA with combinations of these enzymes yielded fewer than 10 restriction fragments. Each of the \textit{PacI-SwaI}, \textit{PmeI-SwaI}, and \textit{PacI-PmeI} digests generated eight restriction fragments with one band shown to consist of two fragments. By using a low voltage electric field, a long separation time, and a wide range of pulse times, we were able to separate large DNA fragments of more than 2000 kb in size employing the PFGE apparatus. Digestion of genomic DNA with the above mentioned enzymes yielded numbers and distribution...
of DNA fragments suitable for physical mapping [27].

The DNA fragment sizes are summarized in Table 2. When undigested total genomic DNA was run concurrently with the digested DNA using the same condition, no extrachromosomal DNA was visible (data not shown). Therefore, all restriction fragments generated using either PacI, PmeI, or SwaI were considered to be chromosomal DNA.

3.2. Genome size of X. campestris pv. glycines YR32

X. campestris pv. glycines YR32 genomic sizes were estimated from the summation of the individual fragment sizes represented by each restriction endonuclease digestion. Genomic size, from single or multiple digestion with PacI, PmeI, and SwaI, was estimated at approximately 5020 kb (Table 2). This result was in agreement with the results from measurements of AseI and SpeI digests of genomic DNA (Fig. 2). Digestion of X. campestris pv. glycines YR32 genomic DNA with AseI and SpeI yielded approximately 37 and 47 restriction fragments, respectively, with several bands containing more than one DNA fragment. The genome size was estimated to be 4974 ± 123 using SpeI-digested genomic

<table>
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<th>Table 1</th>
<th>Bacterial strains and plasmids</th>
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<td><strong>Strains and plasmids</strong></td>
<td><strong>Relevant characteristics</strong></td>
</tr>
<tr>
<td>E. coli</td>
<td>General host for plasmid propagation</td>
</tr>
<tr>
<td>X. campestris pv. glycines YR32</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Rhodobacter sphaeroides 2.4.1</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
</tr>
<tr>
<td>pH1</td>
<td>31-kb DNA fragment from strain 8ra cloned into pLAFR3</td>
</tr>
<tr>
<td>pMH9</td>
<td>pTZ18U carrying 3-kb DNA fragment containing xpsD</td>
</tr>
<tr>
<td>pXCB2012</td>
<td>pSUP205 carrying three EcoRI DNA fragments containing xanAB, rfbCD, and part of rfbA</td>
</tr>
<tr>
<td>pMK41.1</td>
<td>opsx on 2.3-kb SaI-SsrI fragment from pMK29.28 pUF047</td>
</tr>
<tr>
<td>pHF1.1</td>
<td>2.2-kb BstEII DNA fragment containing 16S and 23S rRNA genes from P. aeruginosa cloned into pH3</td>
</tr>
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DNA, or 4958 ± 60 using AseI-digested genomic DNA.

The apparent absence of plasmids in this bacterium reflects a genomic size equal to the chromosome size. It is possible that any endogenous plasmid(s) were in a supercoiled form and might be too small in size such that it ran out of the gel and could not be detected. However, our previous results [26] indicated that linear or supercoiled plasmid with a size larger than 10.5 kb was routinely detected by running undigested genomic DNA in PFGE. Moreover, considering the standard deviation in the genomic DNA fragment sizing in this study, smaller plasmid(s) with sizes less than 10.5 kb would be insignificant in the approximate calculation of the chromosome size. X. campestris pv. glycines YR32 chromosome is slightly larger than the E. coli chromosome [1], but smaller than the Pseudomonas aeruginosa PAO chromosome [19].

3.3. Ordering the PmeI and SwaI fragments

Several methods employing PFGE are available for the construction of physical maps [15,18]. By using 2-D PFGE and multiple digestion approaches in conjunction with Southern hybridization analysis, we were able to determine the order of PmeI and SwaI fragments (Fig. 3). SwaI cleaved the PmeI-A fragment into two subfragments (Fig. 3A, lane 2), whose sizes corresponded to the 1400- and 53-kb fragments in PmeI-SwaI digests of genomic DNA (Fig. 3A, lane 7), suggesting that this PmeI fragment contained one SwaI site. The PmeI-B fragment was cleaved by SwaI into two subfragments (Fig. 3A, lane 3), whose sizes corresponded to the 1325- and 275-kb fragments in PmeI-SwaI digests of genomic DNA. Separation of PmeI-D and -E fragments after digestion with SwaI did not yield additional subfragments, suggesting that these two PmeI fragments contained no SwaI sites (Fig. 3A, lanes 5 and 6). PmeI digestion of the SwaI-C and -D fragments yielded similar results, suggesting that these two SwaI fragments contained no PmeI sites (Fig. 3A, lanes 10 and 11).

PmeI cleaved the 1950-kb SwaI-A fragment into 1400- and 275-kb subfragments (Fig. 3A, lane 8).

Table 2

<table>
<thead>
<tr>
<th>Enzyme(s)</th>
<th>Sizes (kb) of DNA fragments</th>
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<tbody>
<tr>
<td>PaeI</td>
<td>2600 (2602 ± 42), 2170 (2169 ± 30), 250 (248 ± 11)</td>
</tr>
<tr>
<td>PaeI-SwaI</td>
<td>1950 (1958 ± 30), 1315 (1314 ± 29), 675 (673 ± 17), 400 (405 ± 18), 250 (249 ± 12), 110 (110 ± 9), 70 (70 ± 10)</td>
</tr>
<tr>
<td>SwaI</td>
<td>1950 (1954 ± 33), 1565 (1565 ± 21), 1325 (1325 ± 20), 110 (112 ± 8), 70 (68 ± 6)</td>
</tr>
<tr>
<td>PaeI-SwaI</td>
<td>1930 (1398 ± 30), 1325 (1325 ± 28), 1035 (1035 ± 11), 530 (527 ± 15), 275 (275 ± 8), 90 (88 ± 6), 70 (70 ± 7)</td>
</tr>
<tr>
<td>PaeI</td>
<td>1930 (1933 ± 45), 1600 (1602 ± 31), 1125 (1125 ± 15), 513 (510 ± 27), 90 (90 ± 7)</td>
</tr>
<tr>
<td>PaeI-PaeI</td>
<td>1650 (1655 ± 38), 1125 (1123 ± 25), 675 (674 ± 14), 110 (110 ± 28), 275 (272 ± 12), 250 (248 ± 9), 90 (90 ± 6)</td>
</tr>
</tbody>
</table>

*Total genome size from summation of either single or double digestion fragments was (5020 ± 112) kb.

1. Number in parentheses is the actual value from two different measurements.

2. The largest PaeI fragment size was determined by 2-D PFGE.

3. A doublet.
The sum of these two subfragments was 1675 kb, not 1950 kb, indicating that there should be at least one additional fragment totalling 275 kb (data from lanes 4 and 6 indicating that there were two 275-kb subfragments in PmeI-SwaI digests of genomic DNA). This observation, together with the band intensity of the 275-kb subfragment in EtBr-stained gels (Fig. 3A, lane 8), suggested that PmeI actually cleaved the SwaI-A fragment into three subfragments, i.e. 1400-, 275-, and 275-kb, instead of two. Combining the previous data, we were able to conclude that PmeI-B and -D fragments were linked. The PmeI-C fragment was cleaved by SwaI into 1035- and 90-kb subfragments (Fig. 3A, lane 4), and PmeI cleaved the SwaI-B fragment into 1035- and 53-kb subfragments (Fig. 3A, lane 9), suggesting that the PmeI-C and SwaI-B fragments were overlapping. This was supported by Southern hybridization analysis using the xpsD gene probe (data not shown). The probe hybridized strongly to both PmeI-C, SwaI-B, and the 1035-kb subfragment seen in PmeI-SwaI digests of genomic DNA. Previously, it was shown that the PmeI-A fragment was cleaved by SwaI into 1400- and 53-kb subfragments. Together these data demonstrated that PmeI-A and -C fragments were linked.

Although subfragments derived from the PmeI digest of SwaI-B fragment were too faint to be seen in the photo gel (Fig. 3A, lane 9), they were visible in the negative film of the gel. It was deduced from these results that PmeI cleaved the SwaI-B fragment into 1035- and 53-kb subfragments. Summation of these two subfragments was in agreement with the size of the SwaI-B fragment. Previous analysis showed that SwaI cleaved the PmeI-A fragment into 1400- and 53-kb subfragments. These data show that the SwaI-B fragment overlapped the PmeI-A fragment. Since both SwaI-A and -B fragments contained the same 1400-kb subfragment, it is indicated that SwaI-A and -B fragments are closely linked.

One 275-kb subfragment of the SwaI-A fragment was also shown to be associated with the 1325-kb subfragment of PmeI-B fragment, suggesting that the SwaI-A and PmeI-B fragments overlap. Since the 1325-kb SwaI fragment was not cleaved by PmeI, this suggests that the SwaI-A and -C fragments are closely linked. We concluded from these data that the SwaI-A, -B, and -C fragments were linked together in the order C-A-B, or vice versa.

The same-sized 1400-kb subfragment was seen in digestions of the PmeI-A fragment with SwaI (Fig. 3, lane 2) and the SwaI-A fragment with PmeI (Fig. 3, lane 8), suggesting that these two fragments were overlapping. Evidence from Southern hybridization analysis using a pat gene as a probe (Fig. 4) con-
confirmed this conclusion. The probe hybridized strongly to both PmeI-A (Fig. 4, lane 3), SwaI-A (Fig. 4, lane 4), and the 1400-kb subfragment seen in PmeI-SwaI digests of genomic DNA (Fig. 4, lane 6). The PmeI digest of the SwaI-A fragment also contained two 275-kb subfragments (Fig. 4, lane 8), the order of which was unknown. Since the sequence of the SwaI-A, -B, and -C fragments has been determined, the positions of PmeI-A, and -B-D fragments relative to those three SwaI fragments could be determined. The PmeI-A fragment (Fig. 4, lane 2) must overlap the SwaI-B fragment (Fig. 4, lane 9) since both these fragments contained the same 53-kb subfragment. This ordering was in agreement with the result that the PmeI-A fragment was linked to the PmeI-C fragment, which contained a 1035-kb subfragment of the SwaI-B fragment. These data indicated that the SwaI-C fragment should be located upstream of PmeI-A-C fragments, and therefore the fragment must overlap the PmeI-B fragment. A 90-kb subfragment was derived from the SwaI digest of PmeI-C fragment (Fig. 4, lane 4). Since PmeI did not cleave the 90-kb SwaI-D fragment, the data suggested that the SwaI-D fragment was located within the PmeI-C fragment (Fig. 4, lane 11). From the previous data, we were able to determine the order of the PmeI restriction fragments to be -B-D-A-C-E, which overlaps the ordering of the SwaI restriction fragments -C-A-B-D-E (Fig. 6).

3.4. Ordering the PacI fragments

PacI digested X. campestris pv. glycines YR32 genomic DNA genomic DNA into three restriction fragments (Fig. 1). The order of the PacI-A, -B, and -C fragments relative to those PmeI fragments was determined using multiple digestion of PacI-PmeI digests of genomic DNA, 2-D PFGE analysis and Southern hybridization analysis, as previously described for the PmeI and SwaI fragments. We also determined the order of the PacI fragments relative to those of SwaI. Any ambiguities that appeared in 2-D PFGE analysis were resolved by Southern hybridization analysis using selectable genetic markers (Table 1) and by multiple digestion of X. campestris pv. glycines YR32 genomic DNA.

Physical mapping of the X. campestris pv. glycines YR32 chromosome has revealed a genomic structure that consists of a single circular chromosome. There was no evidence of any extrachromosomal DNA larger than 10.5 kb in this strain.
3.5. Constructing a partial genetic map

Southern hybridization experiments were used to localize previously described genes of *X. campestris* (Table 1). The *pat* (Fig. 4) and *opsX* probes hybridized strongly to the *PacI*-A, *PmeI*-A, and *SwaI*-A fragments. It also hybridized to the 1650-kb subfragment of *PacI*-PmeI, the 1400-kb subfragment of *PmeI*-SwaI, and the 1950-kb subfragment of *PacI*-SwaI digests of genomic DNA. The *xpsD, xanAB*, and *rfbCDAB* probes (data not shown) hybridized strongly to the *PacI*-B, *PmeI*-C, and *SwaI*-B fragments. The probes also hybridized to the 1125-kb subfragment of *PacI*-PmeI, the 1035-kb subfragment of *PmeI*-SwaI, and the 710-kb subfragment of *I-CeuI*-PacI digests of genomic DNA. Further Southern hybridization analysis with *I-CeuI* single and double digests of genomic DNA suggested that these genes were located within the larger *I-CeuI* fragment, the 1565-kb subfragment of *I-CeuI*-PacI, the 800-kb sub-fragment of *I-CeuI*-PmeI, and the 710-kb subfragment of *I-CeuI*-SwaI digests of genomic DNA (Fig. 6).

All genetic markers used in this work are thought to be involved in plant pathogenesis (Table 1). The *pat* gene, a DNA fragment conserved in several plant and animal pathogens [9], was located on the same fragment as *opsX*, a gene which is involved in lipopolysaccharide biosynthesis and encodes a virulence factor [11]. *xpsD*, whose gene product is required for the secretion of extracellular enzymes across the outer membrane of *X. campestris* [8], was located on the same fragment as *rfbCDAB* and *xanAB* genes (see Fig. 6). *rfbCDAB* is a cluster of genes involved in the synthesis of the dTDP-rhamnose unit necessary for lipopolysaccharide production, while the *xanAB* gene encodes proteins involved in synthesis of the UDP-glucose and GDP-mannose units required for xanthan gum production [12]. Localization of other pathogenesis genes will be required in order to explain any possible correlation between genome structure and the distribution of pathogenesis genes on the chromosome.

3.6. Two rRNA genes localization employing intron-encoded *I-CeuI* restriction endonuclease

To determine the number of rRNA genes and their locations on the chromosome, we utilized an intron-encoded restriction endonuclease *I-CeuI*, which recognizes a highly conserved 19-bp sequence in *rrl* genes for the large rRNA subunit (23S) [15]. Digestion with *I-CeuI* and other enzymes determined the precise location of the 23S rRNA genes on the chromosome (Fig. 5). *I-CeuI* cleaved *X. campestris pv. glycines* YR32 genomic DNA into two fragments (Fig. 5, lane 2) with the smaller fragment estimated at approximately 430 kb. Based on previous results
[14,15], the data from this experiment indicated that *X. campestris* pv. *glycines* YR32 has two 23S rRNA genes, and they are 430 kb away from each other. From the estimated genome size, we therefore calculated the larger fragment to be 4590 kb in size.

The *PacI*-B fragment was cleaved by *I-Ceu* into three subfragments, whose sizes were approximately 1565, 430, and 175 kb (Fig. 5, lane 3). The *PacI*-A and -C fragments appeared undigested in *I-Ceu*-*PacI*-digested genomic DNA, suggesting that these two fragments did not contain 23S rRNA genes. The data indicate that the two 23S rRNA genes are located within the *PacI*-B fragment. *I-Ceu* did not cleave the *Pmel*-B, -D, and -E fragments (Fig. 5, lane 4), suggesting that these three *Pmel* fragments also did not contain *I-Ceu*-cut sites. *I-Ceu* cleaved the *Pmel*-A fragment into 1825- and 105-kb subfragments, and the *Pmel*-C fragment into 800- and 325-kb subfragments. The summation of the 105-kb subfragment of *Pmel*-A and the 325-kb subfragment of *Pmel*-C, which totals approximately 430 kb, clearly indicated that one 23S rRNA gene was located within the *Pmel*-A fragment, and the other was within the *Pmel*-C fragment.

Digestion of *SwaI* fragments with *I-Ceu* yielded seven fragments (Fig. 5, lane 5). *I-Ceu* did not cleave the *SwaI*-A, -B, -D, and -E fragments, indicating that these four fragments do not have any *I-Ceu* sites. The *SwaI*-C fragment was cleaved by *I-Ceu* into 715-, 430-, and 175-kb subfragments, with the latter two appearing as a single intensely stained band. These data showed that the two rRNA genes were located within the *SwaI*-C fragment. Southern hybridization analysis using a fragment derived from the *P. aeruginosa* rRNA operon as a probe has confirmed these results (data not shown). This probe hybridized to *Pmel*-A and -C fragments, to the 1035- and 530-kb subfragments of the *Pmel*-*SwaI* digests, and to the 1315-kb subfragment of *PacI*-*SwaI* digests of genomic DNA.

Multiple digestions of *I-Ceu* genomic DNA fragments with two different enzymes, i.e. *I-Ceu*-*PacI*-*Pmel* (Fig. 5, lane 6), *I-Ceu*-*Pmel*-*SwaI* (Fig. 5, lane 7), and *I-Ceu*-*PacI*-*SwaI* digests (Fig. 5, lane 8), also confirmed the positions of the 23S rRNA genes. This analysis clearly supports the positions of the 23S rRNA genes and verifies the total genome size of *X. campestris* pv. *glycines* YR32.

The finding that *X. campestris* pv. *glycines* (and perhaps all *X. campestris* pathovars) have only two 23S rRNA genes deserves special attention. Bacteria can contain up to 10 repeated *rrn* operons within their genomes [4,13]. Data correlating bacterial genome size suggest that there is no significant relationship between the number of *rrn* operons and genome size. The fast-growing *E. coli*, with a genome slightly smaller than *X. campestris*, has seven *rrn* operons [1], and *Bacillus subtilis* with a genome of approximately 4.1 Mb has 10 *rrn* operons [14], while *P. aeruginosa* with a genome relatively larger than *X. campestris* has four *rrn* operons [18].

This chromosome map will serve as a framework in our efforts to refine the physical map of *X. campestris* pv. *glycines* YR32, to localize transposon insertion sites and genes involved in pathogenesis, and to study the dynamics or the genome plasticity of this agriculturally important bacterium.

Acknowledgments

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