

oriC Region and Replication Termination Site, *dif*, of the *Xanthomonas campestris* pv. *campestris* 17 Chromosome

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A 13-kb DNA fragment containing *oriC* and the flanking genes *thdF*, *orf900*, *yidC*, *rnpA*, *rpmH*, *oriC*, *dnaA*, *dnaN*, *recF*, and *gyrB* was cloned from the gram-negative plant pathogen *Xanthomonas campestris* pv. *campestris* 17. These genes are conserved in order with other eubacterial *oriC* genes and code for proteins that share high degrees of identity with their homologues, except for *orf900*, which has a homologue only in *Xylella fastidiosa*. The *dnaA/dnaN* intergenic region (273 bp) identified to be the minimal *oriC* region responsible for autonomous replication has 10 pure AT clusters of four to seven bases and only three consensus DnaA boxes. These findings are in disagreement with the notion that typical *oriCs* contain four or more DnaA boxes located upstream of the *dnaA* gene. The *X. campestris* pv. *campestris* 17 *attB* site required for site-specific integration of cloned fragments from filamentous phage ϕ Lf replicative form DNA was identified to be a *dif* site on the basis of similarities in nucleotide sequence and function with the *Escherichia coli dif* site required for chromosome dimer resolution and whose deletion causes filamentation of the cells. The *oriC* and *dif* sites were located at 12:00 and 6:00, respectively, on the circular *X. campestris* pv. *campestris* 17 chromosome map, similar to the locations found for *E. coli* sites. Computer searches revealed the presence of both the *dif* site and XerC/XerD recombinase homologues in 16 of the 42 fully sequenced eubacterial genomes, but eight of the *dif* sites are located far away from the 6:00 point instead of being placed opposite the cognate *oriC*. The differences in the relative position suggest that mechanisms different from that of *E. coli* may participate in the control of chromosome replication.

The gram-negative plant pathogen *Xanthomonas campestris* pv. *campestris* is the causal agent of black rot in crucifers, causing tremendous loss in agriculture worldwide (48). This bacterium is also known to produce an exopolysaccharide, xanthan gum, which is important in pathogenicity and has a variety of applications in oil drilling, food, cosmetics, and agriculture (37). We have previously constructed a physical map of the *X. campestris* pv. *campestris* 17 chromosome (4.8 Mb) bearing restriction sites for four rare-cutting enzymes, *Swa*I (6 sites), *Pac*I (5 sites), *I-Ceu*I (2 sites), and *Pme*I (2 sites), and determined the chromosomal location for 23 genetic loci, including two rRNA operons and the genes involved in xanthan synthesis and yellow pigmentation (42). However, the map still lacks important landmarks, such as the origin of chromosome replication (*oriC*) and the sequence equivalent to that of *Escherichia coli dif* required for resolution of chromosome dimers (2, 5, 10, 20).

Bacterial chromosome replication is initiated at the *oriC* region where a cluster of four or more DnaA boxes are present for the binding of DnaA, an initiator protein sharing high degrees of sequence similarity to homologues from different bacteria, to cause local unwinding of DNA within *oriC* (8, 20, 22, 31, 53). The *oriC* region can be isolated as autonomously replicating DNA fragments, called *oriC* plasmids (16, 17, 18, 21, 28, 34, 36, 54, 56). Studies of *oriC* regions from more than

10 species of eubacteria have revealed several important characteristics (*Bacillus subtilis*, accession number NC_000964; *Bacillus halodurans*, NC_002570; *Buchnera* sp. APS, NC_002528; *Clostridium acetobutylicum*, NC_003030; *Pasteurella multocida*, NC_002663; *Pseudomonas aeruginosa*, AE_004091; *Pseudomonas putida*, X62540; *Staphylococcus aureus* Mu50, NC_002578; and *Xylella fastidiosa*, NC_002488). First, genome organization of the *oriC* region is conserved. In the *oriC* flanking regions, *gidA*, *thdF*, *yidC*, *rnpA*, *rpmH*, *dnaA*, *dnaN*, *recF*, and *gyrB* have been identified, and clustering of *dnaA*, *dnaN*, *recF*, and *gyrB* is common. Second, in gram-positive bacteria, DnaA boxes exist in both upstream and downstream noncoding regions flanking *dnaA* gene and replication initiates within the downstream DnaA box region; in contrast, the DnaA box cluster is only found upstream of the *dnaA* gene in gram-negative bacteria (22). However, different organizations have also been observed in some other bacteria. The *oriC* regions of *E. coli*, *Vibrio harveyi*, and other enteric bacteria are located between *gidA* and *mioC* far upstream from the *dnaA* gene (56). *Caulobacter crescentus oriC* is located between *hemE* and *rpsT* about 2 kb away from *dnaA*, which is separated from the *dnaN*, *recF*, *gyrB* cluster by a distance of 150 kb (55). The *dnaA* gene is not present in the vicinity of the *Coxiella burnetii oriC* region, which contains only two DnaA boxes (36). In *Sinorhizobium meliloti*, *Synechocystis* spp., and *Prochlorococcus marinus*, *oriC*-like sequences have not been detected in the regions next to that of *dnaA* (18, 26, 27).

In *E. coli*, the circular chromosome replicates in a bidirectional manner, and replication forks meet in the terminus region directly opposite *oriC* (1, 10). Lying in the middle of the

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terminus region is the *dif* site required for converting circular chromosome dimers into monomers at the end of a replication cycle (2, 5, 10). Strains deleted for *dif* are incapable of normal partitioning of the newly replicated chromosomes into daughter cells, resulting in the Dif phenotype, which includes abnormal nucleoid morphology, filamentation in a fraction of the cells, induction of the SOS repair system, and decreased growth rate and plating efficiency compared to those of wild-type cells (10, 23). It is known that RecA function and the RecBCD and RecF pathways contribute to the formation of chromosome dimers. The Dif phenotype is suppressed in a *recA* mutant, partially suppressed by either of the mutations in *recB* or *recF*, and completely suppressed by the double mutations (10, 35).

In *X. campestris* pv. *campestris*, a chromosomal *attB* site has been identified which is required for the site-specific integration of the cloned replicative form (RF) DNA fragment from the filamentous phage ϕ Lf (15). In this study, we (i) cloned and sequenced the *X. campestris* pv. *campestris* 17 *oriC* region, (ii) showed that the *attB* site also served as the *dif* site required for chromosome dimer resolution, and (iii) determined the location of *oriC* and *dif* sites on the chromosome map.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) broth and Luria agar were the media for cultivation of *E. coli* (37°C) and *X. campestris* (28°C). The antibiotic used was ampicillin (50 μ g/ml), chloramphenicol (34 μ g/ml), gentamicin (15 μ g/ml), kanamycin (50 μ g/ml), or tetracycline (15 μ g/ml).

DNA techniques. Restriction endonucleases and other enzymes were purchased from New England Biolabs (Beverly, Mass.), Takara (Kyoto, Japan), and Amersham (Uppsala, Sweden) and were used according to the instructions provided by the suppliers. The procedures described by Sambrook et al. (29) were used for preparation of chromosome and plasmid DNA, agarose gel electrophoresis, end labeling of oligonucleotides, preparation of 32 P-labeled probes, Southern hybridization, and transformation of *E. coli*. Nucleotide sequence was determined by the dideoxy chain termination method of Sanger et al. (30). Electroporation was performed to transform *X. campestris* as previously described (45). A DNA fragment internal to the *X. campestris* pv. *campestris* 17 *dnaA* gene was PCR amplified with the *X. campestris* pv. *campestris* 17 chromosome as the template and with primers UP1 (5'-GAAGAGTTTTTCACACC TT-3'), corresponding to nucleotides (nt) 1412 to 1429, and DOWN1 (5'-GCA CCGTTCGTATGGTCGC-3'), complementary to nt 878 to 859 from the start codon of *P. putida dnaA*, respectively (7). These two regions represent the most conserved regions among the bacterial *dnaA* genes.

Tagging of *oriC* and *dif* sites and pulsed-field gel electrophoresis (PFGE). To localize the *oriC* region on the *X. campestris* pv. *campestris* 17 chromosome, the gene tagging method developed previously (42) was employed to insert additional rare-cutting sites, one for *SwaI* and one for *PacI*, near the *oriC* region. The steps included the following: (i) cloning of the 1.3-kb *KpnI-EcoRI* fragment containing the C terminus of *thdF* gene and the N terminus of *orf900*, from the leftmost part of the pXK130 insert (Fig. 1A) into pUC18 to generate pUEK1.3; (ii) cloning of the *NotI* segment containing the rare-cutting sites and the kanamycin resistance gene (*Km^r*) from pUT-Tn5(pfm)CmKm (49) into the *NotI* site within the pUEK1.3 insert, resulting in pUEK1.3Km; and (iii) electroporation of pUEK1.3Km into *X. campestris* pv. *campestris* 17, allowing for chromosomal integration via the homologous region, the 1.3-kb *KpnI-EcoRI* fragment. The mutant strain obtained was designated Xc17-ori. The same strategy was used to tag the *X. campestris* pv. *campestris* 17 *dif* site, except that the *NotI* segment containing the rare-cutting sites and the *Km^r* gene from pUT-Tn5(pfm)CmKm was cloned into pFSK to generate pKFSK (Table 1), which was then electroporated into *X. campestris* pv. *campestris* 17, allowing for integration into the *dif* site and generating strain Xc17::KFSK.

The procedures described by Tseng et al. (42) were used for the preparation of intact chromosomes, in-gel digestion of the chromosomes with *PacI* and *SwaI*, and PFGE in a CHEF-DR III machine from Bio-Rad (Richmond, Calif.).

Analysis of DNA and protein sequences. Sequence analysis was performed with the Genetics Computer Group (Madison, Wis.) package. The BLAST algorithms of the National Center for Biotechnology Information were used for sequence searches. The complete genome sequences of eubacteria were from NCBI Microbial Genomes, <http://www.ncbi.nlm.nih.gov/cgi-bin/entrez/genom-table-cgi>.

Nucleotide sequence accession number. The nucleotide sequence of the *oriC*-containing region from *X. campestris* pv. *campestris* 17 has been registered in GenBank under accession number AY057934.

RESULTS

Cloning of the *X. campestris* pv. *campestris* 17 *oriC*-containing fragment. To clone the *oriC*-containing fragment from *X. campestris* pv. *campestris* 17, an internal fragment of the *X. campestris* pv. *campestris* 17 *dnaA* gene was synthesized by PCR, cloned, and used as a probe for Southern hybridization to screen for positive clones in an *X. campestris* pv. *campestris* 17 genomic library. The primers used for PCR (described in Materials and Methods) represented two of the most conserved regions within the *dnaA* gene of *P. putida* that has a genomic G+C content similar to that of the *Xanthomonas* genomes (63 to 67%) (4). Nucleotide sequence determined for this fragment (571 bp) showed 64% identity to that of the corresponding region of *P. putida dnaA* (7). This fragment was cloned into pBSK+ to generate plasmid pBA571. Using the labeled 571-bp fragment as the probe for Southern hybridization with the library constructed by cloning the fragments generated by partial digestion with *Sau3A1* into pOK12, several clones showing hybridization signals were obtained. One of the clones, designated pXK130 and containing an insert of 13 kb, was capable of replication in *X. campestris* pv. *campestris* 17, indicating that the insert contained the *X. campestris* pv. *campestris* 17 *oriC* gene. Figure 1A shows the restriction map of the pXK130 insert.

Sequence of the *X. campestris* pv. *campestris* 17 *oriC*-containing fragment. The 13-kb insert of pXK130 was subcloned into M13mp18 and mp19, and the nucleotide sequences of both strands were determined. Sequence analysis revealed 12,837 bp containing nine open reading frames (ORFs). These ORFs, with the first five running leftward and the last four running rightward, were identified by sequence similarity shared with the amino acids encoded by the genes clustered with *oriC* in other bacteria (except for *orf900*). Contiguously, these genes were *thdF* (nt 729 to 1, incomplete), *orf900* (nt 3454 to 752), *gidC* (nt 5268 to 3547), *rnpA* (nt 5730 to 5284), *rpmH* (nt 5909 to 5769), *dnaA* (nt 6153 to 7481), *dnaN* (nt 7755 to 8855), *recF* (nt 9685 to 10860), and *gyrB* (nt 10974 to 12837, incomplete) (Fig. 1A). Table 2 shows the percent identities of the amino acid sequences shared with those of the homologues from *X. fastidiosa*, *P. putida*, *P. aeruginosa*, *E. coli*, and *B. subtilis*. Among the homologues, the *X. fastidiosa* proteins always shared the highest degrees of identity with those of *X. campestris* pv. *campestris* 17, ranging from 50 to 93% (Table 2).

There were three large intergenic regions, which were between *rpmH* and *dnaA* (243 bp), *dnaA* and *dnaN* (273 bp), and *dnaN* and *recF* (829 bp).

The *X. campestris* pv. *campestris* 17 minichromosome. To localize the minimal region of *X. campestris* pv. *campestris* 17 chromosome required for autonomous replication, subfragments from the pXK130 insert were generated by either re-

TABLE 1. Bacterial strains, plasmids, and phage used in this study

Strain or plasmid	Genotype or description	Source or reference
<i>E. coli</i> DH5 α	<i>endA1 hsdR17</i> ($r_{K^{-}}$ $m_{K^{+}}$) <i>supE44 thi-1 recA gyrA relA1</i> ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)	Bethesda Research Laboratories
<i>Xanthomonas</i>		
<i>X. campestris</i> pv. <i>campestris</i> 17	A wild-type strain isolated in Taiwan, Ap ^r	50
Xc17-ori	<i>X. campestris</i> pv. <i>campestris</i> 17 derivative with <i>SwaI-PacI</i> sites and Km ^r gene integrated near <i>oriC</i> , Ap ^r , Km ^r	This study
Xc17::KFSK	<i>X. campestris</i> pv. <i>campestris</i> 17 derivative with <i>SwaI-PacI</i> sites and Km ^r gene integrated at the <i>dif</i> site, Ap ^r , Km ^r	This study
L1	<i>X. campestris</i> pv. <i>campestris</i> 17 <i>recA</i> mutant constructed by inserting a Km ^r cartridge into the <i>HindIII</i> site of the <i>recA</i> gene, Ap ^r , Km ^r	12
NT3	<i>X. campestris</i> pv. <i>campestris</i> 17 <i>recA</i> mutant constructed by inserting a Gm ^r cartridge into the <i>HindIII</i> site of the <i>recA</i> gene, Ap ^r , Gm ^r	This study
NT1(Δ 4445)	<i>X. campestris</i> pv. <i>campestris</i> 17 mutant constructed by replacing the 4,445-bp FHR with a Tc ^r cartridge, Ap ^r , Tc ^r	15
NT2(Δ 345)	<i>X. campestris</i> pv. <i>campestris</i> 17 mutant constructed by replacing the 345-bp <i>HincII</i> (containing a <i>dif</i> site) fragment within FHR with a Tc ^r cartridge, Ap ^r , Tc ^r	5
NT1R(att51)	Derived from NT1(Δ 4445) by reinserting the <i>dif</i> site, carried by a pDIFT insert (a 51-bp <i>HincII-PstI</i> fragment), via homologous recombination at the Tc ^r gene, Ap ^r , Tc ^r	15
NT1 <i>recA</i> ::Gm	Derived from NT1(Δ 4445) by inserting a Gm ^r cartridge into the <i>recA</i> gene, Ap ^r , Tc ^r , Gm ^r	This study
Xc17NT1::HIMA	Derived from NT1(Δ 4445) by inserting pDIFHIM into the <i>himA</i> gene, Ap ^r , Tc ^r , Km ^r	This study
Xc17NT1::XHC	Derived from NT1(Δ 4445) by inserting pDIFXHC into the UDP-glucose dehydrogenase gene, Ap ^r , Tc ^r , Km ^r	This study
Plasmid		
pOK12	<i>E. coli</i> general cloning vector, P15A replicon, <i>lacZ</i> α fragment, Km ^r	43
pBluescript+	<i>E. coli</i> general cloning vector, ColE1 replicon, <i>lacZ</i> α fragment, Ap ^r	Stratagene
pUC18	<i>E. coli</i> general cloning vector, ColE1 replicon, <i>lacZ</i> α fragment, Ap ^r	51
pUC19G	Gm ^r cartridge from pUCGM ligated with the blunt-ended <i>AvaII-SspI</i> large fragment from pUC19	This study
pUC4K	Derivative of pUC18, providing a Km ^r cartridge	39
pUCGM	Derivative of pUC18, providing a Gm ^r cartridge	32
pHP45 Ω -Tc	Derivative of pBR322, providing a Tc ^r cartridge	24
pBA571	An internal fragment of <i>X. campestris</i> pv. <i>campestris</i> <i>dnaA</i> (571 bp) amplified by PCR and cloned in pOK12	This study
pXK130	A 13-kb <i>Sau3A1</i> fragment containing <i>oriC</i> from <i>X. campestris</i> pv. <i>campestris</i> cloned in pOK12	This study
pXK12	The upstream 9,471-bp <i>KpnI-HindIII</i> fragment from a pXK130 insert cloned in pOK12, autonomous replication in <i>X. campestris</i> pv. <i>campestris</i>	This study
pUEK1.3	The upstream 1,310-bp <i>KpnI-EcoRI</i> fragment from a pXK130 insert cloned in pUC18	This study
pUEK1.3Km	<i>NotI</i> fragment containing <i>SwaI-PacI</i> sites and a Km ^r gene from pUT-Tn5(pfm)CmKm cloned into pUEK1.3	This study
pXB7	The downstream 5,537-bp <i>HindIII-BamHI</i> fragment from a pXK130 insert cloned in pOK12	This study
pUP1	The <i>PstI</i> fragment from a pXK130 insert (nt 6231–7242) cloned in pUC19G	This study
pUP2	The <i>PstI</i> fragment from a pXK130 insert (nt 4432–6231) cloned in pUC19G	This study
pOH1.7	The <i>HindIII</i> fragment from a pXK130 insert (nt 7706–9450) cloned in pOK12	This study
pGORI2333	A PCR fragment corresponding to nt 5527–7859 of a pXK130 insert cloned in pUC19G, autonomous replication in <i>X. campestris</i> pv. <i>campestris</i> 17 ^a	This study
pGORI1041	A PCR fragment corresponding to nt 6819–7859 of a pXK130 insert cloned in pUC19G, autonomous replication in <i>X. campestris</i> pv. <i>campestris</i> 17 ^a	This study
pGORI547	A PCR fragment corresponding to nt 7313–7859 of a pXK130 insert cloned in pUC19G, autonomous replication in <i>X. campestris</i> pv. <i>campestris</i> 17 ^a	This study
pFSK	pBluescript+ derivative carrying a 792-bp <i>PstI-NotI</i> insert, containing an <i>attP</i> site, from phage ϕ Lf that can mediate site-specific integration into the <i>X. campestris</i> pv. <i>campestris</i> 17 chromosome	This study
pKFSK	pFSK derivative with the <i>NotI</i> fragment containing <i>SwaI-PacI</i> sites and a Km ^r gene from pUT-Tn5(pfm)CmKm inserted in the <i>NotI</i> site	This study
pDIF	The 51-bp <i>HincII-PstI</i> segment containing the <i>X. campestris</i> pv. <i>campestris</i> 17 <i>dif</i> site cloned in pOK12	This study
pDIFT	pDIF derivative with a Tc gene inserted in the <i>HindIII</i> site of the multicloning sites of the vector	This study
pBSU327	A 2.7-kb fragment containing the <i>X. campestris</i> pv. <i>campestris</i> 17 <i>recA</i> gene cloned in pUC18	12
pDIFXHC	A 0.6-kb <i>XhoI-HincII</i> fragment containing part of the <i>X. campestris</i> pv. <i>campestris</i> 17 UDP-glucose dehydrogenase gene cloned in pDIF	This study
pDIFHIM	A 0.4-kb <i>KpnI-BamHI</i> fragment containing the <i>X. campestris</i> pv. <i>campestris</i> 17 <i>himA</i> gene cloned in pDIF	This study

^a pGORI2333, pGORI1041, and pGORI547 each carried a PCR fragment amplified by using forward primers corresponding to nt 5527 to 5544, 6819 to 6838, and 7313 to 7331 of the pXK130 insert, respectively, and the same reverse primer with a sequence complementary to nt 7842 to 7859 of the pXK130 insert.

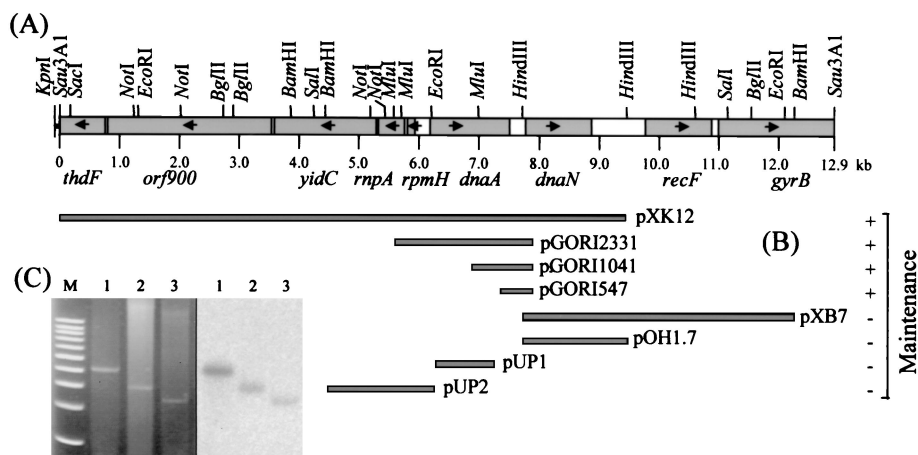


FIG. 1. (A) Restriction map of the pXK130 insert, a *Sau3A1* partial fragment, from *X. campestris* pv. *campestris* 17 containing *oriC* and the flanking genes. The restriction sites were deduced from the nucleotide sequence. The leftmost *KpnI* site was from the vector. (B) Deletion mapping of the *oriC* region. The *oriC*-containing fragments were obtained by either restriction enzyme digestion or PCR amplification and were cloned in the *E. coli* vector pOK12 (pXK12, pXB7, and pOH1.7) or pUC19G (pUP1, pUP2, pGORI2331, pGORI1041, and pGORI547), as described in Table 1. A plus or minus indicates that the plasmid was capable or incapable of autonomous replication in *X. campestris* pv. *campestris* 17, respectively. (C) Detection of minichromosomes in *X. campestris* pv. *campestris* 17. Plasmids pGORI2331 (lane 1), pGORI1041 (lane 2), and pGORI547 (lane 3) were extracted from the *X. campestris* pv. *campestris* 17 transformants and treated with *EcoRI*, which cut the vector once. The linearized plasmids were subjected to agarose gel electrophoresis (left panel) and were transferred by Southern blotting, followed by hybridization with labeled pGORI547 as the probe (right panel).

striction cut or PCR amplification. The subfragments were then cloned into pOK12 (Km^r) or pUC19G (Gm^r) (Table 1, Fig. 1B). The resultant plasmids were separately electroporated into the *X. campestris* pv. *campestris* 17 *recA* mutants, L1 (for pUC19G derivatives) and NT3 (for pOK12 derivatives), and were selected for kanamycin or gentamicin resistance on LB agar plates. Since the vector could not be maintained, kanamycin or gentamicin resistance suggested that the insert, a minichromosome, contained the *oriC* which could support autonomous replication. Alkaline lysis was performed to detect the plasmids, and the plasmids extracted were subjected to Southern hybridization. The ability of autonomous maintenance was then further confirmed by transformation of *X. campestris* pv. *campestris* 17 cells with the same plasmids. Four of the clones containing overlapping inserts as shown in Fig. 1B, pXK12 (9,471-bp insert), pGORI2333 (2,333-bp insert), pGORI1041 (1,041-bp insert), and pGORI547 (547-bp insert), were found to be maintained autonomously as shown by Southern hybridization with labeled pGORI547 extracted from *E. coli* DH5 α (pGORI547) as the probe (Fig. 1C). Among these *oriC*-containing plasmids, pGORI547 possessed the smallest

insert that included the C-terminal 169 bp of *dnaA*, the 273-bp *dnaA/dnaN* intergenic region, and the N-terminal 105 bp of *dnaN* (Fig. 1B and 2A). We therefore identified the 273-bp *dnaA/dnaN* intergenic region as the *oriC* of *X. campestris* pv. *campestris*. pGORI2333 containing the complete *dnaA* gene showed a higher intensity of hybridization signal than the other plasmids with truncated *dnaA*, indicating that a higher copy number of pGORI2333 was present (Fig. 1C). To test maintenance, L1 cells containing pGORI2333, pGORI1041, or pGORI547 were subcultured five times in overnight cultures with gentamicin, each of which underwent about five doublings, and equal amounts of the cells were subjected to plasmid extraction. Ethidium bromide-stained plasmid DNA bands with intensities comparable to those from the respective plasmids of the original clones were observed (data not shown). These data indicated that these autonomous plasmids could be maintained with the pressure of antibiotics in the *recA* background.

DnaA boxes and the pure AT clusters in the *X. campestris* pv. *campestris* 17 *oriC* region. Sequence analysis of the 273-bp *X. campestris* pv. *campestris* 17 *oriC* revealed some important

TABLE 2. Percent identities of deduced amino acid sequences from the genes around *X. campestris* pv. *campestris* 17 *oriC* with those of the corresponding gene products from other bacteria

Bacterium ^a	% Identity for protein:								
	ThdF ^b	ORF900	YidC	RnpA	RpmH	DnaA	DnaN	RecF	GyrB ^b
<i>X. fastidiosa</i> (NC_002488)	71	57	69	50	93	83	77	59	83
<i>P. putida</i> (X62540)	58	— ^c	40	37	75	49	55	33	66
<i>P. aeruginosa</i> (NC_002516)	62	—	39	35	80	48	55	33	65
<i>E. coli</i> (L10328)	56	—	36	35	78	54	50	34	67
<i>B. subtilis</i> (NC_000964)	38	—	—	37	63	38	26	27	53

^a Accession numbers are in parentheses.

^b Only a partial sequence is available.

^c —, no homologue present.

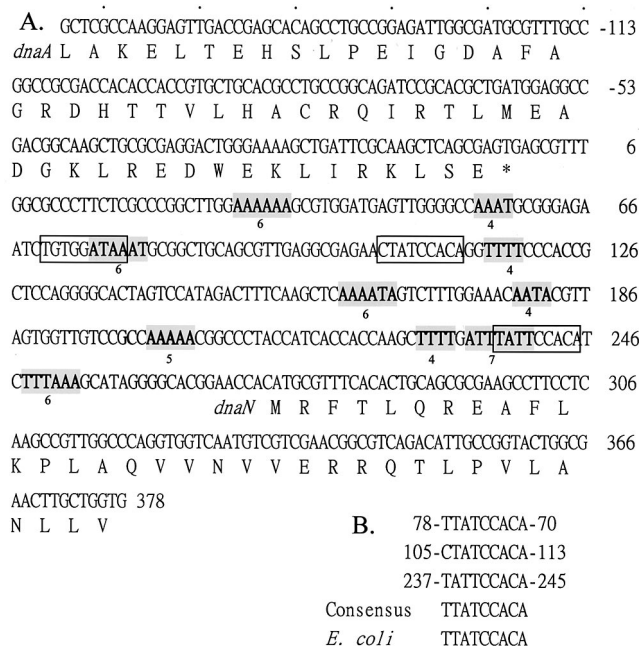


FIG. 2. (A) Nucleotide sequence of the 547-bp fragment containing the *X. campestris* pv. *campestris* 17 *oriC* region. The base after the *dnaA* stop codon is counted as nt 1. Shown are the C terminus of *dnaA*, the 273-bp intergenic region, and the N terminus of *dnaN*. The three DnaA boxes are boxed, and the AT-rich clusters are shaded and in bold, each with an Arabic numeral to indicate the number of bases. (B) Nucleotide sequences of the three predicted *X. campestris* pv. *campestris* 17 DnaA boxes (the first one is the sequence on the complementary strand) and the consensus sequence of the *E. coli* DnaA box.

characteristics. First, it contained 49% A+T, which was much higher than that of the *Xanthomonas* chromosomes (33 to 36% A+T) (4). However, no AT-rich repeats resembling that in *oriC* of *E. coli* and other bacteria were found; instead, 10 pure AT clusters were present: one 7-mer, four 6-mers, one 5-mer, and four 4-mers (Fig. 2A). Second, it contained only three DnaA boxes which had a consensus sequence identical to that of *E. coli* (Fig. 2A and B) (31). They were situated within the *dnaA/dnaN* intergenic region, at bp 70 to 78, bp 105 to 113, and bp 237 to 245 downstream of the *dnaA* termination codon (Fig. 2A). The same relative location was found only in *X. fastidiosa* *oriC* (296 bp long; located in the *dnaA/dnaN* intergenic region), which contains five DnaA boxes and a very high A+T content (72%, including two long strings of AT-rich clusters) compared to that of the whole genome (52.5%) (21). However, no nucleotide sequence identity was shared between these two *oriCs*.

Chromosomal location of the *X. campestris* pv. *campestris* 17 *oriC* region. The *X. campestris* pv. *campestris* 17 *ori* gene was localized by the gene tagging strategy described in Materials and Methods, in which one *PacI* and one *SwaI* site carried by a suicide plasmid were integrated into the 1.3-kb *KpnI-EcoRI* fragment containing the C terminus of the *thdF* gene and the N terminus of *orf900* (Fig. 1A), resulting in the tagged mutant Xc17-ori. To compare the restriction patterns, chromosomes from *X. campestris* pv. *campestris* 17 and Xc17-ori were separately cut with *PacI* or *SwaI*. Then the digests were separated

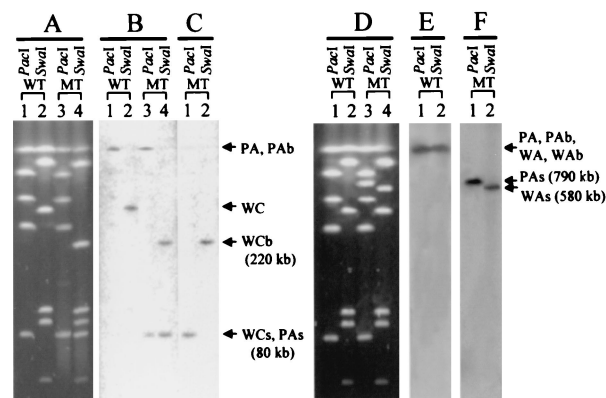


FIG. 3. Southern hybridization of the *SwaI* and *PacI* fragments from the *X. campestris* pv. *campestris* 17 chromosomes. The chromosomes from *X. campestris* pv. *campestris* 17 (wild type [WT] in panels A and D), the *oriC*-tagged mutant Xc17-ori (MT in panel A), and the *dif*-tagged mutant Xc17::KFSK (MT in panel D) were digested with *SwaI* or *PacI* as indicated above the lanes. The digests were subjected to PFGE (A and D). The DNA fragments were transferred onto nylon membranes and hybridized with the ³²P-labeled probes. Both membranes were reused and washed before being probed with another probe. Probes used were pUEK1.3, carrying the 1.3-kb *KpnI-EcoRI* fragment which contains the C terminus of *thdF* gene and the N terminus of *orf900* (B); pOH1.7, carrying the 1.7-kb *HindIII* fragment which contains the *dnaN* gene (C); the 345-bp fragment containing *dif* (E); and pBSU327, containing the *recA* gene (F). Designations of the important fragments and the hybridizing signals are indicated at the right-hand side of each set of panels.

by PFGE, which displayed five *PacI* fragments and six *SwaI* fragments (Fig. 3A, lanes 1 and 2) with known sizes (42). Using labeled pUEK1.3, which contains the 1.3-kb *KpnI-EcoRI* insert, as the probe for Southern hybridization with the digests of the *X. campestris* pv. *campestris* 17 chromosome, the hybridization signal was found to associate with PA (*PacI* fragment A, 3,270 kb) and WC (*SwaI* fragment C, 300 kb) (Fig. 3B, lanes 1 and 2), indicating that *oriC* was located within the PA/WC overlapping region (Fig. 4). This overlapping region has previously been calculated to be 160 kb (42). The PA fragment from the Xc17-ori chromosome was cut by *PacI* into subfragments PAb and PAs. Since PAs and PE (*PacI* fragment E, 80 kb) formed a double band (Fig. 3A, lane 3) as judged by the stronger intensity of PAs than that of PE alone in the *X. campestris* pv. *campestris* 17 digest, we estimated the size of PAs to be 80 kb. This result suggested that the *X. campestris* pv. *campestris* 17 *oriC* region was located ca. 80 kb from the PA/PE interface (Fig. 4). After *SwaI* digestion, the WC fragment of the Xc17-ori chromosome was missing and two subfragments, WCb (220 kb) and WCs (80 kb), were generated (Fig. 3A, lane 4). This result suggested that the *oriC* region was located ca. 80 kb from the WA/WC interface and ca. 220 kb from the WC/WD interface (Fig. 4). Here, the distance from *oriC* to the WA/WC interface plus that from *oriC* to the PA/PE interface were equivalent to the length of the PE/WC overlapping region. In other words, the *oriC* region was located in the middle of the PA/WC overlapping region. Southern hybridization with the labeled plasmid pUEK1.3 as the probe showed signals with PAb, PAs, WCb, and WCs (Fig. 3B, lanes 3 and 4), confirming that *X. campestris* pv. *campestris* 17 *oriC* was located within the PA/WC overlapping region (Fig. 4). Taking

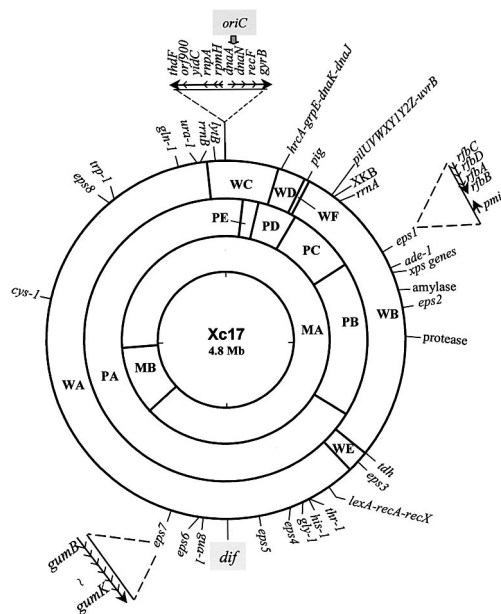


FIG. 4. Improved chromosome map of *Xanthomonas campestris* pv. *campestris* 17. The map originally constructed bore 13 rare-cutting sites (*Pme*I, 2; *Pac*I, 5; and *Swa*I, 6) and 23 genetic loci (42). Additional loci determined in this study include, in clockwise order, the *oriC* region; *hrcA*, *grpE*, *dnaK*, and *dnaJ*, the stress-responsive genes (47); *pilUVWXYZ* and *uvrB*, the type IV pilus biogenesis genes and DNA repair gene (13); *xps* gene cluster, the type II protein secretion gene cluster; the amylase gene; the protease gene; the *dif* site; and the *lytB* homologue. Arrows outside the circles represent the transcriptional directions. Gene location and transcriptional direction were determined by PFGE separation of the chromosomal digests in conjunction with Southern hybridization as described in this and other studies (13, 42).

this data together, we set the middle point in the PA/WC overlapping region as 12:00 on the circular *X. campestris* pv. *campestris* 17 chromosome map.

To determine the orientation of the genes clustered with *X. campestris* pv. *campestris* 17 *oriC*, Southern hybridization was performed with the digests from the Xc17-*ori* chromosome by using the probe prepared from plasmid pOH1.7 that carries the 1.7-kb *Hind*III fragment containing the *dnaN* gene (Fig. 1A and B). The hybridization signal was associated with PAs and WCb (Fig. 3C), indicating that the *dnaN* gene localized to the right of *oriC* on the *X. campestris* pv. *campestris* 17 map (Fig. 4). In other words, the five left-side genes (*thdF*, *orf900*, *ydC*, *rmpA*, and *rpmH*) were oriented counterclockwise, and the four right-side genes (*dnaA*, *dnaN*, *recF*, and *gyrB*) were oriented clockwise (Fig. 4).

Identification of the *X. campestris* pv. *campestris* 17 *dif* sequence. Previously we demonstrated that cloned fragments from the filamentous phage ϕ Lf RF DNA can mediate integration, either by homologous recombination at the ϕ Lf-homologous region (FHR) in the *X. campestris* pv. *campestris* 17 chromosome or by site-specific integration at the *attB* site within the FHR (15). The FHR is 4,445 bp long and is composed of three consecutive *Hinc*II fragments of 2,266, 1,834, and 345 bp, with the *attB* site being contained in the 345-bp fragment. Requirement of the FHR region for integrations was

demonstrated by using two deletion mutants, NT1(Δ 4445) and NT2(Δ 345), in which the FHR and the 345-bp *attB*-containing *Hinc*II fragment, respectively, were replaced with a Tc^r cartridge. Both types of integration were abolished in NT1(Δ 4445), whereas only the site-specific integration was impaired in NT2(Δ 345) (15). It was also demonstrated that the mutant NT1R(*att51*), in which the 51-bp *Hinc*II-*Pst*I segment (within the 345-bp fragment) containing the *attB* site was reinserted into the Tc^r cartridge of NT1(Δ 4445), could still accommodate the wild-type levels of site-specific integration (15). With striking similarity to *E. coli dif*, the *X. campestris* pv. *campestris* 17 *attB* site contained an identical central core, 8 of 11 nucleotides identical left arm and 8 of 11 nucleotides identical right arm (15). Because of the homology and because one of the consequences caused by *dif* deletion is the appearance of a subpopulation of stringed cells, a deletion-induced filamentation called the Dif phenotype (13), we were interested to know whether deletion of the *attB* site in *X. campestris* caused the same consequence. In this study, NT1(Δ 4445) and NT2(Δ 345) were used to test for the effects. In both mutant strains cultured in LB broth, over 40% of the cells were filamentous as revealed by microscopy (Fig. 5B and C). In contrast, microscopic examination of NT1R(*att51*) showed that the wild-type morphology was restored (Fig. 5A and D). Based on these results, we proposed that the *attB* site for ϕ Lf integration possessed the function required for the resolution of the chromosome dimer. It was therefore called *dif* for *X. campestris*, following the designation for *E. coli*.

In *E. coli*, RecA function is involved in the formation of chromosomal dimers and mutation of *recA* suppresses the Dif phenotype (10, 35). In this study, the *recA* gene of NT1(Δ 4445) was knocked out by gene replacement with the Gm^r cartridge, and the resultant double mutant was designated NT1*recA*::Gm. The Dif phenotype of this strain was found to be suppressed (data not shown).

Chromosomal location of the *X. campestris* pv. *campestris* 17 *dif* sequence. To localize the *dif* site on the circular *X. campestris* pv. *campestris* 17 chromosome map, we used the same tagging strategy as that for localizing *oriC*, except that the fragment used to mediate integration was the 792-bp *Pst*I-*Not*I fragment carrying the *attP* site from ϕ Lf RF DNA. The resultant tagged strain was called Xc17::KFSK. In Southern hybridization with the digests of *X. campestris* pv. *campestris* 17 chromosome using the labeled 345-bp fragment as the probe, the hybridization signal was associated with fragments PA (*Pac*I fragment A, 3,270 kb) and WA (*Swa*I fragment A, 2,880 kb), indicating that the *dif*-like sequence resided within the overlapping region of fragments WA and PA (Fig. 3E). In the digests of the Xc17::KFSK chromosome, fragments WA and PA were cut by *Swa*I and *Pac*I into WAb plus WAs (580 kb) and PAb plus PAs (790 kb), respectively. With labeled pBSU327 carrying an insert containing the *recA* gene (Fig. 4) (12) as the probe for Southern hybridization, the signal was associated with PAs and WAs (Fig. 3F), indicating that the *dif* sequence was located 790 kb from the PA/PB interface and 580 kb from the WA/WE interface (Fig. 4). On the basis of the data obtained from determination of the chromosomal locations, the distance between *oriC* and *dif* could therefore be calculated by summing up the sizes of WAs, WCb (220 kb), and the remaining *Swa*I fragments (whose sizes had been known pre-

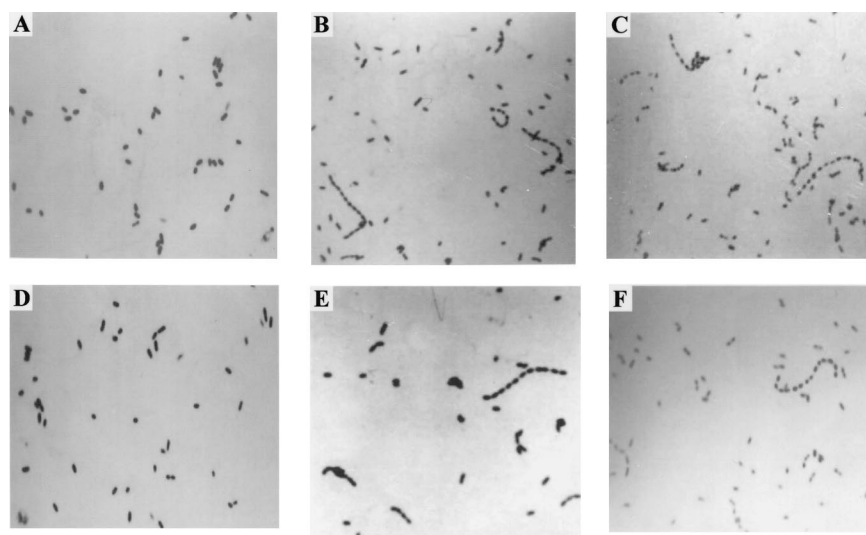


FIG. 5. Effects of deletion and reinsertion of the *dif* site on the cell morphology of *X. campestris* pv. *campestris*. (A) Wild-type *X. campestris* pv. *campestris* 17; (B) NT1(Δ 4445), deleted for the 4,445-bp FHR; (C) NT2(Δ 345), deleted for the 345-bp *Hinc*II fragment within FHR; (D) NT1R(att51), the *dif* site reinserted near the original site; (E) Xc17NT1::HIMA, the *dif* site inserted in the *himA* gene; (F) Xc17NT1::XHC, the *dif* site inserted in the UDP-glucose dehydrogenase gene. Cells were observed under a light microscope.

viously): WD (125 kb), WF (17 kb), WB (1,369 kb), and WE (98 kb) (Fig. 4) (42). The sum was 2,409 kb, approximately half the size of the 4.8-Mb *X. campestris* pv. *campestris* 17 chromosome (42). In other words, the chromosomal locations of the *X. campestris* pv. *campestris* 17 *dif* site and *oriC* were on opposite sides (Fig. 4), like those of *E. coli* (20).

In *E. coli*, the location of the *dif* site is important; the Dif phenotype can be reverted by reinsertion of the *dif* sequence at a region near the original site, around 30 kb (14, 23, 40). In this study the effects of the location of the *dif* sequence on the *X. campestris* pv. *campestris* 17 chromosome were evaluated in mutants NT1::XHC and NT1::HIMA. NT1::XHC and NT1::HIMA were constructed by inserting plasmid pDIF, which carries the 51-bp *Hinc*II-*Pst*I fragment containing the *dif* site, into *eps3*, carrying the UDP-glucose dehydrogenase gene (near 4:30, ca. 640 kb from the original *dif* site) and the *himA* gene (near 7:00, immediately upstream of *eps7*, which contains the *gum* gene cluster ca. 415 kb from the original *dif* sequence), respectively (Fig. 4). Microscopic examination showed that the Dif phenotype was not reverted by either of the insertions (Fig. 5E and F).

Distribution of *dif*-like sequences in bacteria. To detect the distribution of *dif*-like sequences in *Xanthomonas*, the chromosomal DNA from *X. campestris* pathovars citri, manihotis, phaseoli, and vesicatoria and *X. oryzae* pv. *oryzae* were digested with *Eco*RI, separated in agarose gel, and subjected to Southern hybridization with the probe prepared from pDIF that carries the 51-bp *Hinc*II-*Pst*I fragment containing the *dif* site. A single hybridization band was observed in each of the digests, indicating the presence of a *dif* sequence in all the *Xanthomonas* strains tested (data not shown).

The occurrence of *dif*-like sequences and XerC/XerD recombinase homologues, the so-called Xer site-specific recombination system (XRS), has been described for several bacteria. In addition, 42 eubacterial genomic sequences have been published (as of 16 October 2001). In this study we searched

the databases containing these genomes for nucleotide sequences similar to that of the *E. coli dif* site (the 28-bp region) and XerC/XerD homologues (3) by using E values of 10 and -30 , respectively, as the cut points. Only 16 of these genomes, representing those from 13 species, were found to have both *dif* sequence and XerC/XerD homologues (Fig. 6). When the chromosomal location of the *dif* sequence relative to that of cognate *oriC* (setting the region containing DnaA boxes as 12:00) was compared, it was found that only eight of them, *B. halodurans* C-125, *B. subtilis*, *E. coli* K-12, *E. coli* O157:H7, *E. coli* O157:H7 EDL933, *S. aureus* Mu50, *S. aureus* N315, and *Vibrio cholerae* had a location close to 6:00, between 5:32 and 6:24 (Fig. 6). Five others were near either 4:00 (*N. meningitidis* MC58, *N. meningitidis* Z2491, and *P. aeruginosa*) or 8:00 (*C. acetobutylicum* and *X. fastidiosa*) (Fig. 6). The remaining three

Bacterium	<i>dif</i> sequence	O'clock	Accession number
<i>Xanthomonas campestris</i>	acttcgcataa gtata tatgtcagaa	~6:00	AY057934
<i>Bacillus halodurans</i> C-125	ggttctataa atata tatgtaaa	6:24	NC_002570
<i>Bacillus subtilis</i>	acttctaga atata tatgtaaa	5:32	NC_000964
<i>Clostridium acetobutylicum</i>	gaagactataa ggata tatgttaaa	8:02	NC_003030
<i>Escherichia coli</i> K-12	ggtgcgcaaa gtata tatgttaaa	5:53	NC_000913
<i>Escherichia coli</i> O157:H7	ggtgcgcaaa gtata tatgttaaa	6:19	NC_002695
<i>Escherichia coli</i> O157:H7 EDL933	ggtgcgcaaa gtata tatgttaaa	6:07	NC_002655
<i>Haemophilus influenzae</i> Rd	atttcgcaaa ataaa tatgttaaa	9:40	NC_000907
<i>Neisseria meningitidis</i> MC58	agttcgcaaa gtata tatgttaaa	4:06	NC_003112
<i>Neisseria meningitidis</i> Z2491	agttcgcaaa gtata tatgttaaa	4:14	NC_003116
<i>Pasteurella multocida</i> PM70	acttcgcataa gtata tatgttaaa	3:25	NC_002663
<i>Pseudomonas aeruginosa</i> PAO1	gattcgcataa gtata tatgttaaa	4:17	NC_002516
<i>Staphylococcus aureus</i> Mu50	acttctataa atata tatgtaaa	6:05	NC_002758
<i>Staphylococcus aureus</i> N315	acttctataa atata tatgtaaa	5:54	NC_002745
<i>Ureaplasma urealyticum</i>	gaaggaa aa gtata gatgtaaa	3:39	NC_002162
<i>Vibrio cholerae</i>	agtgcgt at at gtat gt tat gt taaa	6:19	NC_002505
<i>Xylella fastidiosa</i>	agcggacataa atgca tatgcgaaa	8:00	NC_002488
	gatggacataa atgca tatgtaaa	8:00	NC_002488
Consensus sequence	agttcgcaaa gtata tatgttaaa		

FIG. 6. The *dif* sequences of the 17 eubacteria with known genome sequences (except for *X. campestris* pv. *campestris* 17) and their positions relative to the DnaA boxes of the cognate *oriC* at 12:00. The 6-nt central cores are boxed, and the conserved bases are shaded. Shown at the bottom is the consensus sequence derived from the alignments.

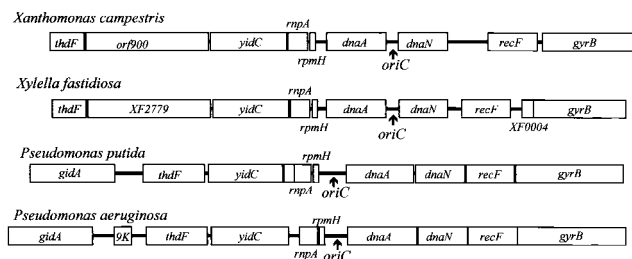


FIG. 7. Conservation of the local gene order around the *oriC* region of *X. campestris* pv. *campestris* 17, *X. fastidiosa* (GenBank accession number NC_002488), *P. putida* (X62540), and *P. aeruginosa* (NC_002516).

bacteria, *Haemophilus influenzae* Rd, *Pasteurella multocida*, and *Ureaplasma urealyticum* had *dif* sequences located at 9:40, 3:25, and 3:39, respectively (Fig. 6). In the latter eight cases, the *dif* sequences were located far away from the 6:00 point, with distances ranging from 147 kb (in *U. urealyticum*) to 689 kb (in *P. aeruginosa*). Finally, we also noted that (i) *X. fastidiosa* contained two *dif*-like sequences, which were about 10 kb apart (Fig. 6) (33), and (ii) *V. cholerae* had two chromosomes, but only the larger one possessed a *dif* sequence (9).

DISCUSSION

In this study we have characterized the *oriC* and *dif* sites of *X. campestris* pv. *campestris* 17. Identification of *oriC* was based on the observations that (i) the cloned fragments containing the *dnaA/dnaN* intergenic region (273 bp) were capable of autonomous replication, (ii) three DnaA boxes and several AT-rich clusters are contained within the 273-bp region, and (iii) the gene order around this region is conserved with those in other bacteria (Fig. 7). The *attB* site previously shown to be the region for site-specific integration of the cloned fragments from ϕ Lf RF DNA (15) was identified as the *dif* site based on the observations that (i) deletion of this region caused filamentation of the cells, (ii) reinsertion of a 51-bp segment containing *attB* near the original site resulted in a restoration of the wild-type phenotype, and (iii) the Dif phenotype was suppressed by a secondary mutation in the *recA* gene (Fig. 5).

Two distinct characteristics of the *X. campestris* pv. *campestris* 17 DnaA boxes are worth noting. First, the *X. campestris* pv. *campestris* 17 *oriC* region contains only three DnaA boxes, which disagrees with the notion that a cluster of four or more DnaA boxes is an indication of a functional chromosome origin (22). Second, the *X. campestris* pv. *campestris* 17 DnaA boxes are located downstream of *dnaA* within the *dnaA/dnaN* intergenic region, a location different from that found for other gram-negative bacteria which are usually situated upstream of the *dnaA*, *dnaN*, *recF*, *gyrB* cluster or detached from *dnaA* (22). The same location relative to *dnaA* has been found only for *oriC* of *X. fastidiosa* (21, 33), a plant pathogenic bacterium shown to be closely related to *Xanthomonas* on the basis of 16S rRNA sequence comparison (46) and on the high degrees of identity shared between the protein homologues of the two bacteria (6). However, no similarity is shared between the nucleotide sequences of the two *oriC* regions.

The presence of *orf900* is special, because only a homologue

of unknown function is found in the corresponding region of *X. fastidiosa* (XF2779) but not in any other bacterial *oriC* regions sequenced to date. The protein product deduced from *orf900* possesses interesting structural features. Amino acid positions 257 to 441 share 30% identity (46% similarity) with plasmid-encoded NodB from *Sinorhizobium meliloti*, a protein of 217 amino acids possessing chito-oligosaccharide deacetylase activity, required for nodulation (41). The same region also shares 36% identity (52% similarity) with the *pgdA* gene encoding a 463-amino-acid peptidoglycan, *N*-acetylglucosamine deacetylase, between amino acids 286 and 441 in *Streptococcus pneumoniae*, proposed to contribute to virulence (44). In addition, amino acid positions 786 to 819, 820 to 853, and 854 to 887 of the *orf900* product are three tandem motifs called the tetratricopeptide repeat, which is a degenerate 34-amino-acid repeated motif found in organisms as diverse as bacteria and humans, where the tetratricopeptide repeat proteins are involved in processes such as cell cycle control, transcription repression, stress response, protein kinase inhibition, mitochondrial and peroxisomal protein transport, and neurogenesis (11). We have knocked out *orf900* by insertional mutation with a gentamicin resistance gene cartridge into the *Bgl*III site within its coding region and have tested for properties including colony morphology, phage sensitivity, and pathogenicity. The preliminary data indicated that no changes had been caused by the mutation (data not shown).

The organization of the genes around the *X. campestris* pv. *campestris* 17 *oriC* region is largely the same as that for *X. fastidiosa*, except that there is an ORF (XF0004) located between *X. fastidiosa* *recF* and *gyrB*. The organization is also highly similar to that of the *P. putida* and *P. aeruginosa* *oriC* regions, except that *orf900* is absent from the later regions and that a small gene (*9K*) is inserted between *yidC* and *rnpA* in *P. aeruginosa* (Fig. 7). Similar genome organization is also found in the fully sequenced genomes of *B. subtilis* (accession number NC_000964), *B. halodurans* (NC_002570), *Buchnera* sp. APS (NC_002528), *C. acetobutylicum* (NC_003030), *P. multocida* (NC_002663), and *S. aureus* Mu50 (NC_002578).

For several bacteria (excluding *E. coli*), incompatibility has been observed between the plasmid *oriC* and chromosomal *oriC* due to titration of the endogenous DnaA protein by the plasmid *oriC* (22). Therefore, the copy number of *oriC* plasmids is usually low (25, 52, 54). In this study, the low copy number of the *oriC* plasmids with truncated *dnaA* indicates that the titration effect may also occur in *X. campestris* pv. *campestris* 17, and the higher copy number of pGORI2333 suggests that additional DnaA might have been produced from the plasmid-borne *dnaA* gene, making the total DnaA sufficient for the use of both *oriC* genes. In some bacteria, the *oriC*-containing plasmids cannot be maintained stably and tend to integrate into the chromosome (19, 21, 34, 38). The integration, mostly at or near their respective *oriC* regions, occurs even when a *recA* strain is used. In contrast, the *X. campestris* pv. *campestris* 17 *oriC*-containing plasmids studied here can be maintained autonomously in the *recA* strain under the selective pressure of an antibiotic.

By using a gene-tagging strategy we have localized *X. campestris* pv. *campestris* 17 *oriC* in the middle of the PA/WC overlapping region, which was therefore set as 12:00 on the circular chromosome map (Fig. 4). By using the same strategy

the *dif* site was localized opposite *oriC*, near 6:00. Thus, the relative positions of *oriC* and *dif* are similar to those for *E. coli*, suggesting that a similar mechanism is employed to control chromosome replication in the two organisms.

Computer searches for the *dif* sequences and the XerC/XerD homologues and comparison of the relative positions between *oriC* and the cognate *dif* sequence have revealed several interesting points. First, of the 42 fully sequenced eubacterial genomes, only 16 possess the XRS, indicating that a system other than XRS is required for the resolution of chromosome dimers in the other bacteria that lack an XRS. Second, of the 17 bacteria (including *X. campestris* pv. *campestris* 17) that contain an XRS, only 9 (representing 7 species) locate their *dif* sequence near the 6:00 point. In the other eight genomes the distances between the *dif* site and the 6:00 point range from 147 kb (in *U. urealyticum*) to 689 kb (in *P. aeruginosa*). They are much longer than the distances that allow the *dif* site to retain the normal function in *E. coli* (around 30 kb), as demonstrated by the *dif* deletion and reinsertion experiments (14, 23, 40). These findings suggest either that the *dif* sites in these eight bacteria are not contained in the terminus region opposite *oriC* and thus their XRS can use a remote *dif* as the substrate or that their terminus regions containing the *dif* site are actually located at regions distant from the 6:00 point. If the latter proposal is true, then the two replication forks must proceed at unequal speeds during bidirectional replication so that they will meet in the terminus region which is far from the 6:00 point. Finally, in *V. cholerae* the *dif* site is only present in the larger chromosome, indicating that another mechanism(s) is available for the smaller chromosome to resolve the dimeric molecules.

Genome sequencing of *X. campestris* pv. *campestris* 17, the strain used in this study, is underway by collaborative efforts of several research groups, including our laboratory in Taiwan. The chromosome map of *X. campestris* pv. *campestris* 17 constructed previously has been improved in this study, and thus the utility of the map has increased. This will greatly benefit our genome sequencing project.

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M.-R. Yen and N.-T. Lin contributed equally to this work.

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