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A Type Ib ParB Protein Involved in Plasmid Partitioning in a Gram-Positive Bacterium▽

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Our current understanding of segregation of prokaryotic plasmids has been derived mainly from the study of the gram-negative bacterial plasmids. We previously reported a replicon of the cryptic plasmid from a gram-positive bacterium, *Leifsonia xyli* subsp. *cynodontis*. The replicon contains a putative plasmid partition cassette including a Walker-type ATPase followed by open reading frame 4 without sequence homologue. Here we reported that the *orf4* gene was essential for maintaining the plasmid stability in *L. xyli* subsp. *cynodontis*. Furthermore, the purified orf4 protein specifically and cooperatively bound to direct repeat sequences located upstream of the *parA* gene in vitro, indicating that *orf4* is a *parB* gene and that the direct repeat DNA sequences constitute a partition site, *parS*. The location of *parS* and the features of ParA and ParB proteins suggest that this plasmid partition cassette belongs to type Ib, representing the first type Ib cassette identified from a gram-positive bacterial plasmid.

Bacterial plasmids maintain their presence within a growing bacterial population depending on different stabilization systems. While high-copy-number plasmids rely on random distribution among daughter cells, the stabilization of low-copy-number plasmids can be achieved through the mechanisms of plasmid multimer resolution, active partitioning, and postsegregational killing (14, 16, 30, 32).

The typical active partitioning cassettes of low-copy-number plasmids are composed of two genes that are organized as an operon and a *cis*-acting DNA sequence, termed the plasmid partition site or centromere-like region (2). The upstream gene to be transcribed gives rise to an ATPase belonging to a superfamily of phylogenetically related proteins that are involved in plasmid and chromosome segregation, which is called the ParA superfamily. The downstream gene encodes a DNA-binding factor called ParB, which interacts directly with the plasmid partition sites that are normally composed of direct or inverted iterated sequences (36). ParA and DNA-binding proteins assemble on the partition site to form a nucleoprotein complex that is required for the directional movement of paired plasmids away from the cell median (18, 31).

The active partitioning systems can be broadly classified into two types depending on the type of ATPase encoded by the upstream gene. Type I ParA contains the Walker-type ATPase motif, while the type II ATPase belongs to the actin/Hsp70 superfamily (14, 23). The type I partition systems are further divided into type Ia and type Ib subgroups based on the features of the ParB protein, the localization of the partition site, and the mechanism of the transcriptional regulation. In comparison with type Ia systems (3, 7, 19, 28, 33), the type Ib ATPases lack the N-terminal DNA-binding domain that appears to be required for autoregulation of the transcription of the active partition operon. The downstream *parB* genes of type Ia and type Ib partitioning systems are phylogenetically unrelated, and normally type Ia ParB proteins are larger than proteins from subgroup Ib (14).

The role of the *parB* gene in partitioning type Ia plasmids has been well characterized (6, 12, 19, 27, 33), while few studies have focused on type Ib partitioning. Several type Ib ParB-like proteins have recently been identified in plasmids TP228 (1, 4, 5, 15, 17), pVS1 (20), pTAR (21), pB171 (10), pVT745 (13), and pRA2 (24). However, these plasmids are exclusively from gram-negative bacteria (11).

Several active partitioning systems from gram-positive bacterial plasmids have been identified previously (9, 22, 26, 35), but very few studies have been carried out to characterize the mechanism of the partitioning. A recent study showed that δ and ω genes located in the streptococcal plasmid pSM19035 constitute a novel partitioning system, although these two genes are transcribed separately (9). In this system, the δ gene encodes a Walker-type ATPase and the ω-encoded protein is a DNA-binding protein that functions as a global regulator of plasmid functions, including replication, copy number control, and postsegregational killing (8).

Leifsonia xyli subsp. *cynodontis*, originally named *Clavibacter xyli* subsp. *cynodontis* subsp. nov., is a gram-positive bacterium isolated from the xylem of Bermuda grass (*Cynodontis dactylon* L. Per.) (25). A cryptic plasmid about 51 kb in size, namely, pCXC100, was harbored by some *L. xyli* subsp. *cynodontis* isolates but not by others (29). The 5-kb replicon of pCXC100, in which a *parA* homologue and a downstream open reading frame (*orf4*) that has no homologues with any ParB sequence are present, has been identified previously (26). In this study, we aimed to study the function of *orf4* in plasmid maintenance. We found that *orf4* is essential for the plasmid stability in *L. xyli* subsp. *cynodontis*. Furthermore, the orf4 protein was expressed in *Escherichia coli* and in vitro purified. This purified protein specifically and cooperatively binds to the direct repeats lo-

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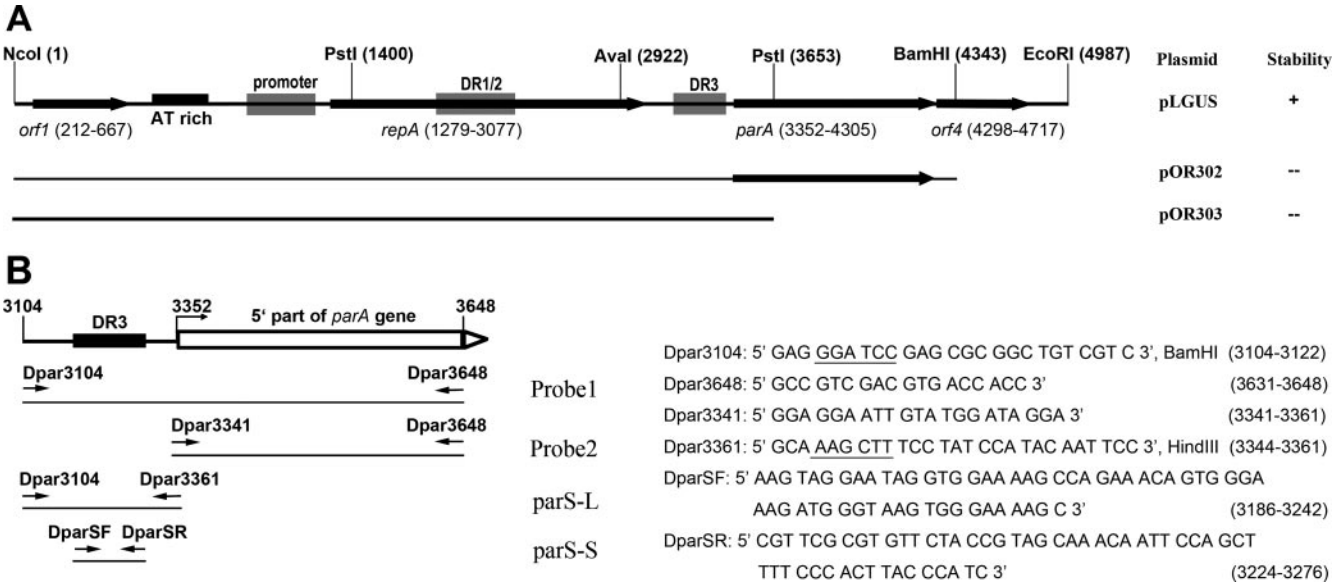

FIG. 1. (A) Physical map of the pCXC100 replicon and the composition of the pCXC100 replicon in the pBR325-based plasmids used in this study. Plasmid pLGUS contains the complete replicon, while pOR302 contains a deleted replicon lacking the majority of *orf4* and pOR303 lacks both the main body of *parA* and the whole *orf4*. Other data shown in this study indicate that *orf4* encodes a ParB analogue that has specific DNA-binding activity. Whether each plasmid stably propagated in *L. xyli* subsp. *cynodontis* is listed on the right: +, the plasmid was stable when propagating in the bacterium; −, the plasmid was not stable. The details of the plasmid stability are shown in Table 1. (B) Probes used in this study to assess the DNA-binding activity encoded by *orf4*. The positions and sequences of primers used for PCR amplification of the corresponding DNA fragments are indicated. Restriction sites for Dpar3104 and Dpar3361, given after the sequences, are underlined. DR, direct repeat.

cated upstream of the *parA* gene, called *parS*. Therefore, the protein encoded by *orf4* is a functional homologue of the ParB protein. The features of *parS* gene products, as well as those of ParA and ParB proteins, further suggest that the active partition cassette of pCXC100 belongs to type Ib.

MATERIALS AND METHODS

Bacteria and plasmids. All *E. coli* strains were grown in LB medium at 37°C. *L. xyli* subsp. *cynodontis* was grown on solid medium (DM agar) at 28°C as previously described (29). Antibiotics for selection of resistant *E. coli* cells were added to final concentrations of 60 μg/ml of ampicillin, 25 μg/ml of chloramphenicol, and 10 μg/ml of tetracycline. Tetracycline at 2 μg/ml was used for selections in *L. xyli* subsp. *cynodontis*. *E. coli* strain DH5α (Gibco BRL) was used as a host for creating subclones of the pCXC100 replicon. *L. xyli* subsp. *cynodontis* strain no. 3, lacking the 51-kb native plasmid, was transformed by plasmid containing derivatives of the pCXC100 replicon by use of an electroporation method (29).

YB411 was constructed for overexpression of the ParB protein. The *parB* gene was amplified by PCR using primers dparB1 (5′-CTG GAA TTC ATG GCT GAT CGC ACG GTT GC; is the EcoRI restriction site; underlined) and dparB2 (5′-GCC AAG CTT AGC TTC CCA GTG GGC GCC; the HindIII restriction site is underlined) and cloned into expression vector pMAL_c2x (New England Biolabs) to produce the maltose-binding protein (MBP)-ParB fusion protein.

pLGUS is a pBR325-based plasmid containing the full pCXC100 replicon (Fig. 1A), which served as the positive control in the plasmid stability assay. This plasmid was constructed as described elsewhere (T.-Y. Li et al., submitted for publication). The plasmids pOR302 and pOR303 were constructed by cloning an NcoI-BamHI (positions 1 to 4343) fragment and an NcoI-PstI (positions 1 to 3653) fragment, respectively, into pBR325 (Fig. 1A). All regular DNA manipulations were carried out by following methods described by Sambrook et al. (34).

Protein expression and purification. MBP and MBP-ParB fusion protein were expressed and purified according to an established protocol (New England Biolabs), with some modifications. The 1-liter culture containing the YB411 plasmid was grown to an optical density at 600 nm of 0.6 to 0.8 and then was induced for 3 h at 37°C by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.4 mM. Cells were harvested by centrifugation and resuspended in 100 ml buffer A (20 mM Tris, pH 8.0, 100 mM NaCl). The collected cells were then sonicated and centrifuged. To remove contamination, the supernatant containing soluble MBP or MBP-ParB protein was loaded onto an SP-Sepharose Fast Flow column (Amersham Pharmacia). The flow fraction was collected and then loaded onto 6-ml amylose columns (New England Biolabs) equilibrated with the same buffer. The unbound proteins were washed away with 20 volumes of the same buffer, and the column-retained products were released by adding 10 mM maltose to the washing buffer. The recovered protein was dialyzed against 1 liter of buffer containing 10 mM Tris-HCl (pH 7.6) and 100 mM NaCl and concentrated by Amicon ultrafiltration (Millipore). A bicinchoninic acid assay kit (Pierce) was used to determine protein concentration.

Plasmid stability assay. Plasmid stability was examined as previously described (26). Plasmid-containing *L. xyli* subsp. *cynodontis* cells were spread and grown on solid DM agar in the absence of antibiotics for 7 days at 28°C, which corresponded to a minimum of 20 generations of growth. Then, cells from the culture lawn were collected, diluted, and spread on fresh solid DM agar to grow for another 7 days free of antibiotics to cumulate the minimum of 40 generations of growth. The subculture was repeated one more time to reach a minimum of 60 generations of growth free of antibiotics. Before the start of growth and after each of the 7-day growth cycles, the culture was diluted and plated on DM agar in the presence or absence of tetracycline to calculate the plasmid retention rate. About 100 colonies were picked up from the antibiotic-free plate to further confirm the plasmid loss (26).

Electrophoretic mobility shift assay. All of the DNA fragments indicated in Fig. 1B were PCR amplified and radiolabeled at the 5′ end with [γ-^{32}P]ATP (5,000 Ci/mmol; Furui Biotech, Beijing, China) by T4 polynucleotide kinase (Promega). The DNA probe (about 15 nM) was incubated with the indicated amounts of the purified proteins in a final volume of 10 μl containing 15 mM Tris-HCl (pH 7.6), 3 mM $MgCl_2$, 0.3 mM dithiothreitol, 50 mM KCl, 1 mM ATP, 0.25 μg poly(dI · dC), and 2% glycerol for 30 min at 30°C, followed by addition of an equal volume of the loading dye containing 50% glycerol and then electrophoresis at 4°C on 5% native polyacrylamide gels containing 5% glycerol in 0.5× Tris-borate-EDTA buffer (0.045 M Tris-borate and 1 mM EDTA at pH 8.0) at ~20 V/cm for about 3 h. Gels were exposed to phosphorimager screens and analyzed by a Typhoo 9200 variable scanner (Amersham Pharmacia).

DNase I footprinting assay. DNA fragment parS-L was amplified by using primers Dpar3104 and Dpar3361 (Fig. 1B) and then cleaved by endonucleases BamHI or HindIII (Takara), and the sticky 5′ end was labeled with [α-^{32}P]dATP (Furui Biotech, Beijing, China) or [α-^{32}P]dCTP (Amersham Pharmacia Biotech, United King-

TABLE 1. Stability of plasmids in *L. xyli* subsp. *cynodontis*[a]

Plasmid (genotype)	Avg retention rate (%) at no. of generations			
	0	20	40	60
pLGUS (*parA orf4*)	100	100	100	100
pOR302 (*parA* Δ*orf4*)	100	96.9	22.8	6.4
pOR303 (Δ*parA* Δ*orf4*)	91.5	78.7	13.8	5.2

[a] The plasmid stability was analyzed as described in Materials and Methods. Two parallel experiments were performed for each plasmid, and the percentages of recovered colonies that contain plasmid are shown.

dom) by Klenow fragment (Takara). The DNA probe was incubated with the indicated amounts of the purified proteins in a final volume of 20 μl at 30°C for 30 min in buffer containing 15 mM Tris-Hcl (pH 7.6), 3 mM $MgCl_2$, 0.3 mM dithisthreitol, 50 nM KCl, 1 mM ATP, and 2% glycerol. After incubation, DNase I (Takara) and 20 μl of the reaction buffer (10 mM $MgCl_2$ and 5 mM $CaCl_2$) were added. Incubation was continued for 3 min, and an equal volume of stop buffer (1% sodium dodecyl sulfate, 200 mM NaCl, and 20 mM EDTA) was added. The reaction mixture was extracted with phenol-chloroform and then ethanol precipitated and resuspended in 8 μl H_2O. The samples were run on a denatured, 8% polyacrylamide sequence gel.

RESULTS AND DISCUSSION

***orf4* is essential for plasmid stability in *L. xyli* subsp. *cynodontis*.** Our previously published results indicate that the *par* locus of pCXC100, containing the putative *parA* gene and *orf4*, is essential for the plasmid stability in *L. xyli* subsp. *cynodontis* (26). No sequence homologue of orf4 has been found from the protein database, and it was unclear whether *orf4* plays a role in maintaining the plasmid stability (26). In this study, we constructed two deletion mutants, one with both *parA* and *orf4* being deleted (pOR303) and the other with *orf4* being deleted (pOR302) (Fig. 1A). In comparison with the plasmid containing the wild-type *par* locus, both deletion mutants resulted in a dramatically decreased stability of the corresponding plasmids when propagated in *L. xyli* subsp. *cynodontis*, and pOR303 was less stable than pOR302 (Fig. 1A; Table 1). These results suggest that *orf4* is essential for stabilizing plasmid in *L. xyli* subsp. *cynodontis*, while *parA* plays an additive role.

The *parA* gene of the pCXC100 *par* locus encodes a typical Walker-type ATPase, indicating that pCXC100 may employ an active partition mechanism to stabilize itself in host cells, consistent with the fact that this cryptic plasmid is a low-copy-

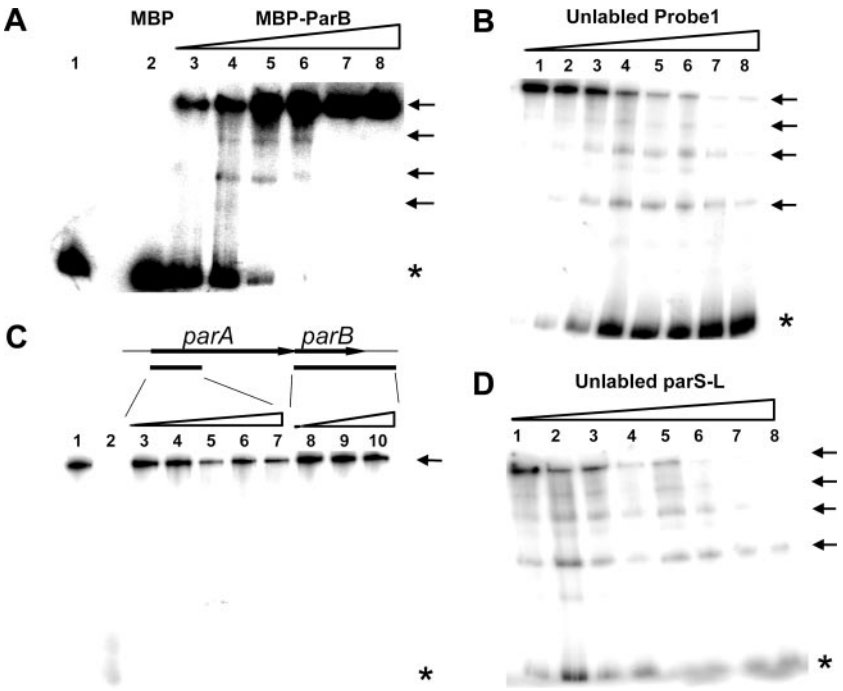

FIG. 2. Electrophoretic mobility shift assay of the specific DNA-binding activity of the ParB protein in vitro. All DNA probes used here were PCR amplified and radiolabeled at the 5′ end with [γ-^{32}P]ATP. Probes are defined in Fig. 1B. (A) Dose-dependent binding of the ParB protein with probe 1. Increasing amounts of MBP-ParB fusion protein (from lane 3 to lane 8, 100, 200, 400, 800, 1,600, and 1,900 ng, respectively) were incubated with a fixed amount of radiolabeled probe 1 DNA fragment. Control samples in lanes 1 and 2 contained 1,000 ng of BSA protein and MBP, respectively. (B) Assay of the competition ability of the unlabeled DNA probe 1 with the labeled probe 1. The ratios of the radiolabeled to unlabeled DNA for the reactions were (from lanes 1 to 8, respectively) 1:0, 1:1, 1:2.5, 1:5, 1:7.5, 1:10, 1:15, and 1:27. (C) Assay of the ability of the unlabeled probe 2 (5′ part of *parA*) and *parB* (containing the full *parB* gene and 280 bp of the downstream sequence) to compete with the labeled probe 1. The ratios of labeled probe 1 to unlabeled probe 2 (from lanes 3 to 7, respectively) were 1:1, 1:5, 1:10, 1:19, and 1:37, and those to the unlabeled *parB* gene (from lanes 8 to 10, respectively) were 1:1, 1:5, and 1:19. Lane 1 contained no unlabeled DNA. The reaction shown in lane 2 was conducted in the same manner as that shown in lane 8 of panel B. (D) Assay of the competition ability of the unlabeled DNA parS-L with the labeled probe 1. The ratios of the radiolabeled to unlabeled DNA were the same as those given for panel B. The amount of MBP-ParB fusion protein for each reaction shown in panels B to D was 200 ng. Asterisks indicate unbound DNA, and arrows indicate different forms of protein-DNA complexes.

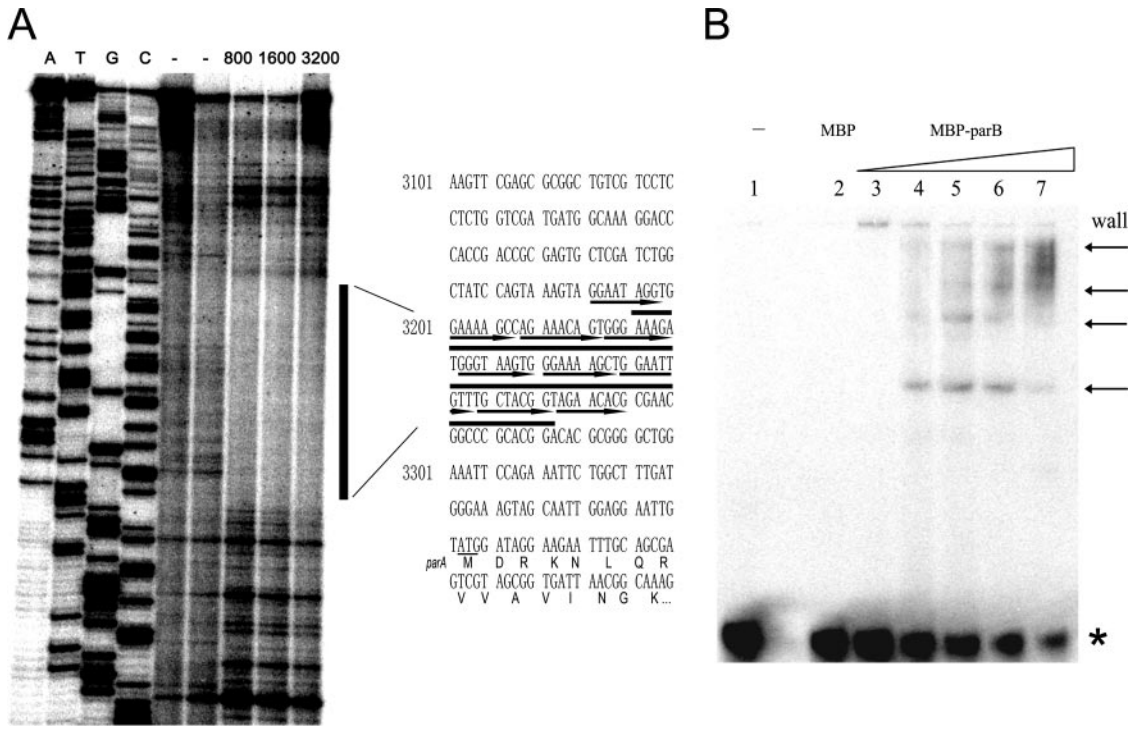

FIG. 3. (A) DNase I footprinting assay of the exact ParB binding sites. The DNA fragment parS-L, labeled at one of the two 5′ ends, was incubated with the indicated amounts of the purified proteins, and the reactions were followed by DNase I cleavage and gel electrophoresis. The negative-control lane (−) on the left contained 2,000 ng BSA, and that on the right contained the same amount of MBP. The amounts of MBP-ParB in the far-right lanes ranged from 800 to 3,200 ng. The vertical black line shows the DNA region protected by the ParB fusion protein, and the protected sequence is shown (overlined) on the right. Black arrows overline the direct repeats (26). DNA ladders generated as described previously (34) are shown on the left lanes. (B) Electrophoretic mobility shift assay of the MBP-ParB fusion protein binding with direct repeat sequences in the AT-rich region in vitro. Lanes 1 and 2 contained 1,000 ng of BSA and MBP, respectively. Lanes 3 to 7 contained 100, 200, 400, 800, and 1,600 ng of the ParB fusion protein, respectively. The asterisk indicates unbound DNA, and arrows indicate different forms of protein-DNA complexes.

number plasmid (26, 29). The *parB* gene is typically located downstream of *parA* in the active partitioning operon. Although no sequence homologue of *orf4* was found within the known *parB* gene, the physical location and function in maintaining the plasmid stability suggest that *orf4* could encode a ParB function.

orf4 specifies a novel ParB protein with sequence-specific DNA-binding activity. The DNA-binding function of orf4 was assessed in vitro by expressing orf4 from the pMAL_C2X expression vector, in which orf4 was fused downstream of MBP. Fusion with MBP both increased the protein solubility and provided a convenient means of purifying the orf4 protein (data not shown).

The ParB protein in an active partitioning cassette is expected to interact directly with the plasmid partition sites that are normally composed of direct or inverted iterated sequences (36). A previous study has shown that an AT-rich region located upstream of the *parA* gene of the pCXC100 replicon contains direct repeat sequences (26), providing a candidate site for orf4 protein binding if it encodes ParB function. Probe 1 (bp 3104 to 3668), covering the AT-rich region and the 5′ part of the *parA* gene, was used to assess the binding activity of orf4 (Fig. 1B). Figure 2A shows that only the fusion protein and not MBP alone bound to the radiolabeled DNA fragment and resulted in DNA species with lower gel mobility, reflecting the formation of DNA-protein complexes. A competition experiment showed that the labeled DNA-protein complex could be competed by the same unlabeled DNA fragment but not by DNA fragments amplified from the *orf4* gene (Fig. 2B and C), indicating that the binding is sequence specific. These results suggest that *orf4* of the pCXC100 *par* locus encodes a novel ParB analogue. This finding agrees with the notion that type Ib ParB lacks a sequence homologue but shares a conserved DNA-binding function and that the diversity of the ParB proteins adds a layer of specificity to the macromolecular interactions inside the nucleoprotein complex driving plasmid segregation (11).

Four shifted DNA species were evident on native gels (Fig. 2A, B, and D), and the most slowly mobilized species corresponding to the largest DNA-protein complex was dominant. This observation raises the possibility that there are at least four ParB binding sites on the DNA sequence and that the fusion protein binds to the DNA in a highly cooperative manner.

To further examine if the ParB binding site *parS* is located solely in the AT-rich region where the direct repeats are present, unlabeled parS-L, containing an AT-rich region, and probe 2, containing the 5′ part of *parA*, were separately added to the competition reaction mixtures. It is shown that the unlabeled probe 2 did not compete with probe 1 for the bind-

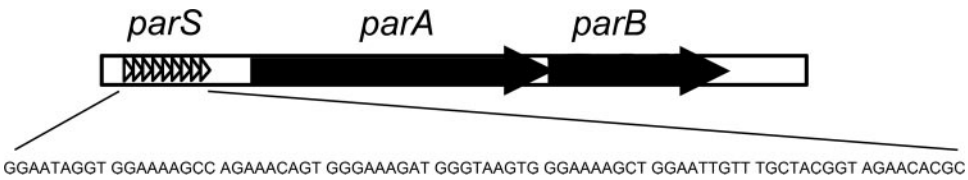

FIG. 4. Overall structure of the active partition cassette (type Ib) of the plasmid pCXC100. Open triangles indicate nine direct repeats located in the partition site (*parS*), and the corresponding sequence is shown below.

ing of ParB at all, while parS-L efficiently competed the binding (Fig. 2C and D), supporting the theory that *parS* is located in the AT-rich region upstream of the *parA* gene.

The partition sites are usually located upstream of the *parA* gene or downstream of the *parB* gene in an active partition cassette (14). However, some cassettes, such as that in plasmid pB171, contain two partition sites, with one being located upstream of the *parA* gene and the other downstream of the *parB* gene (10, 18). The presence of *parS* upstream of the *parA* gene in the pCXC100 partitioning cassette has been demonstrated clearly thus far in this report. Meanwhile, the result shown in Fig. 2C argues against the presence of an additional *parS* gene downstream of *parB*, because the inclusion of the 280-bp sequence downstream of the *parB* gene in the *parB* unlabeled probe showed no activity in competing with the upstream *parS* gene for ParB binding (Fig. 2C, lanes 8 to 10).

***parS* is the direct repeat sequence located upstream of the *parA* gene.** It was shown above that multiple ParB binding sites are present upstream of the *parA* gene. A DNase I footprinting assay was carried out to further examine the ParB binding sequence. The labeled parS-L fragment (Fig. 1B) was incubated with ParB protein and then cleaved by DNase I. It is clearly shown that the direct repeat sequence in the AT-rich region was protected from DNase I nuclease cleavage by MBP-ParB fusion protein but not by MBP or bovine serum albumin (BSA) protein (Fig. 3A; also data not shown), indicating that ParB protein bound to the direct repeats. We noticed that not all of the nine predicted direct repeats were protected from DNase I cleavage by ParB binding (Fig. 3A).

To confirm the results, the direct repeat DNA fragment parS-S (~90 bp) was synthesized (Fig. 1B) (see Materials and Methods) and the ParB binding property of this fragment was studied. Figure 3B shows that ParB effectively and cooperatively bound to this 90-bp fragment, as it did to the large DNA fragment probe 1 (Fig. 2A), and that four similarly shifted bands were evident. We thus concluded that the direct repeat sequences located upstream of the *parA* gene, namely, *parS*, constitute the ParB binding sites (Fig. 4).

The partition cassette of plasmid pCXC100 belongs to type Ib. Clearly, the replicon of pCXC100 has a complete active partition cassette containing a *parS* site upstream of the *parA* gene, and the *parB* gene is downstream of the *parA* gene, with an 8-bp overlap (Fig. 1A and 4). It has been demonstrated that this cassette is responsible for stabilizing the plasmid in its native host *L. xyli* subsp. *cynodontis*, a gram-positive bacterium (26; this study). The feature that ParA is a typical Walker-type ATPase shows that the pCXC100 active partition system belongs to a type I cassette. Furthermore, the ParA protein of the pCXC100 partition cassette has no HTH motif (26), and its ParB protein is short (139 amino acids). Moreover, *parS* of the pCXC100 *par* locus is located upstream of the *parA* gene. This evidence further categorizes the partition cassette of pCXC100 into type Ib. Binding of the ParB protein with the *parS* site is expected to function in both partitioning of the pCXC100 plasmid and autoregulation of the operon expression. Prior to this study, four out of the five reported type Ib *parS* genes have been determined to be direct repeat sequences (11). This study adds a new type Ib *parS* composed of direct repeats (Fig. 4).

In recent years, study of the plasmid partitioning mechanisms has been extended beyond type Ia partition cassettes. For example, the recently studied plasmids from gram-negative bacteria, including plasmid TP228 (1, 4, 5, 15, 17), pVS1 (20), pTAR (21), pB171 (10), pVT745 (13), and pRA2 (24), all contain type Ib partition cassettes (14). However, the partitioning mechanisms utilized by this new type of cassette are unclear. This study reports that the native plasmid pCXC100 from a gram-positive bacterium harbors a type Ib partition cassette. Further characterization of the mechanisms of this partitioning system should provide key insights into the understanding of type Ib plasmid partitioning. Study of the molecular basis for active partitioning of plasmid in *L. xyli* subsp. *cynodontis* should also expand our understanding of chromosome segregation in gram-positive bacteria.

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