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Characterization of the replicon of a 51-kb native plasmid from the gram-positive bacterium *Leifsonia xyli* subsp. *cynodontis*

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Abstract

The 4992-bp replicon of a large cryptic plasmid in the gram-positive bacterium *Leifsonia xyli* subsp. *cynodontis* was identified and sequenced. The replicon encoded two proteins essential for plasmid replication and stability. The putative replication protein (RepA) is homologous to that of the plasmids in mycobacterial pLR7 family, while the putative ParA protein immediately downstream of RepA is significantly homologous to the Walker-type ATPase required for partition of plasmid and chromosome of the gram-positive bacteria. These two proteins and other ORFs are clustered with the putative promoters and other regulatory sequences, illustrating an efficient organization of the replicon for this novel plasmid.
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Keywords: *Leifsonia xyli* subsp. *cynodontis*; pCXC100; Replication; Partition; Gram-positive bacteria

1. Introduction

As an extrachromosomal genetic element, plasmid contains a replicon that automatically replicates the plasmid and controls the plasmid copy number. Several different mechanisms of the plasmid survival and propagation have been reported. These include the multimer resolution system ensuring the plasmid in the monomeric form, the active partitioning system enabling faithful segregation of the plasmid to the daughter cells, the post-segregational killing system resulting in the death of plasmid-free segregants, and the conjugative transfer and mobilization system spreading the plasmid between bacteria [1]. These molecular modules are usually clustered to form the plasmid survival kit, ensuring that the plasmids replicate and spread efficiently [1]. Recent analysis of the enriched data on the plasmid survival mechanisms in different bacteria has identified families of phylogenetically related function distributed across a range of disparate plasmids [2–4]. Supporting the phylogenetic analysis, the *parAB* genes from the chromosome of *Pseudomonas putida* and *Bacillus subtilis* have been found to stabilize the plasmid mini-F in *Escherichia coli* [5,6]. This result suggests that the plasmid and chromosome partitioning systems and the host components involved in plasmid partitioning are highly conserved between gram-positive and gram-negative bacteria.

Several plasmids from gram-positive bacteria have been extensively characterized regarding the mechanisms of replication and stability. For example, pAMβ1 and pLS32 are best known as theta-replicating plasmids [7,8]. Plasmids pCI2000, pSK1 and pAW63 may maintain their stability with active partitioning [9–11]. However, compared to those of the gram-negative bacteria, the studies on the survival mechanisms of plasmids

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from gram-positive bacteria are relatively inadequate [3,9,12]. In this study, we have tried to understand the mechanisms of replication and propagation of pCXC100, a large cryptic plasmid from the gram-positive bacterium *Leifsonia xyli* subsp. *cynodontis*.

Leifsonia xyli subsp. *cynodontis* (*Lxc*), originally named as *Clavibacter xyli* subsp. *cynodontis* subsp. nov., is a gram-positive, high G + C content, coryneform bacterium isolated from the xylem of bermudagrass (*Cynodontis dactylon* L. Per.). It colonizes many crop plants including maize, rice, sorghum, oats, white millet and sudan grass without causing wilting symptoms [13–18]. The broad host range and weak pathogenicity have inspired the idea of using this bacterium as a means for crop protection [18]. However, the extremely slow growth of this bacterium (4–6 h per generation) and the loss of vigor after prolonged time for growth in vitro significantly hinders the study of its genetics [19]. A cryptic plasmid about 51 kb in size, named pCXC100, was harbored by some *L. xyli* subsp. *cynodontis* isolates, but not all [19,20]. Little is known about the mechanism of replication and stability of this plasmid [20]. In this study, the 5 kb DNA replicon of pCXC100 in *L. xyli* subsp. *cynodontis* was cloned and sequenced. Analysis of the sequence revealed that pCXC100 utilizes a complete survival kit, with the genes encoding the replication and propagation proteins being tightly clustered, providing a new system for understanding the survival mechanism of the large cryptic plasmid for the gram-positive bacterium.

2. Material and methods

2.1. Growth and transformation of bacteria

All *E. coli* strains were grown in LB or 2YT medium at 37 °C [21]. *L. xyli* subsp. *cynodontis* was grown on solid medium (DM agar) at 28 °C as previously described [19]. Antibiotics for selections in *E. coli* were added to a final concentration of 50 μg/ml of ampicillin, 25 μg/ml of chloramphenicol, and 10 μg/ml of tetracycline. Chloramphenicol at 5 μg/ml or tetracycline at 2 μg/ml was used for selections in *L. xyli* subsp. *cynodontis. E. coli* strain DH5α (Gibco BRL, Gaitherburg, MD) was used as a host for all subclones of pCXC100. *L. xyli* subsp. *cynodontis* strain #3 lacking the 51 kb native plasmid was transformed by pCXC100 derivatives using electroporation method [19].

2.2. Plasmid isolation from L. xyli subsp. cynodontis and plasmid construction

The miniprep plasmid DNA was prepared from bacterial cells with a modified alkaline extraction procedure as previously described [19], then used to determine the presence of recombinant plasmids transformed to *L. xyli* subsp. *cynodontis*. Large-scale preparations of plasmid DNA for mapping and subcloning were made using CsCl gradient centrifugation [21]. Five plates of culture were harvested in 20 ml of saline and resuspended in 5 ml of 10 mg/ml lysozyme solution, the procedures followed were the same as that of the miniprep after scaling-up. The plasmid band was collected from the CsCl gradient after running at 44,000 rpm for 36 h.

Construction of plasmids containing various regions of the pCXC100 DNA is described in Table 1 as well as in some figures and text. All regular DNA manipulations were carried out following methods described by Sambrook et al. [21].

2.3. Plasmid stability

Due to the difficulty of growing *L. xily* subsp. *cynodontis* in liquid medium, and loss of vigor after prolonged time growth on DM agar solid medium, it is not possible to detect the plasmid stability for many

Table 1
Plasmids used in this study

Name	Description	Host strain
PBR325	ColE-based vector/Cam^r, Ap^r, Tc^r	*E. coli*
PCXC100	Native plasmid in *Lxc*	*Lxc*
pLXC101	pBR325 with 15 kb *Hin*dIII–*Nco*I fragment of pCXC100/Ap^r, Tc^r	*E. coli* and *Lxc*
pLXC102	pBR325 with 9.5 kb *Bgl*II–*Nhe*I fragment of pCXC100/Cam^r, Ap^r	*E. coli*
pLXC103	pBR325 with 22.5 kb *Hin*dIII–*Eco*RI fragment of pCXC100/Ap^r, Tc^r	*E. coli*
pLXC104	pBR325 with 14.5 kb *Hin*dIII–*Bgl*II fragment of pCXC100/Cam^r, Ap^r	*E. coli*
pLXC105	pBR325 with 17 kb *Bgl*II–*Nde*I fragment of pCXC100/Cam^r, Ap^r	*E. coli* and *Lxc*
pLXC106	pBR325 with 8 kb *Hin*dIII–*Bgl*II fragment of pCXC100/Cam^r, Ap^r	*E. coli* and *Lxc*
pLXC107	pLXC101 with internal *Sma*I fragment being deleted/Ap^r, Tc^r	*E. coli* and *Lxc*
pLXC108	pBR325 with 7 kb *Hin*dIII–*Eco*RI fragment of pCXC100/Ap^r, Tc^r	*E. coli* and *Lxc*
pLXC109	pLXC101 with internal *Xho*I fragment being deleted/Ap^r, Tc^r	*E. coli* and *Lxc*
pLXC110	pLXC101 with *Ava*I–*Hin*dIII fragment being deleted/Ap^r, Tc^r	*E. coli* and *Lxc*
pLXC111	pLXC110 with internal *pst*I fragment being deleted/Tc^r	*E. coli*

Note: Lxc stands for *L. xyli* subsp. *cynodontis*.

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generations as it is with other bacterium, like *E. coli*. We tested the plasmid stability in DM agar solid medium for a limit number of generations. Plasmid-containing *L. xyli* subsp. *cynodontis* cells were grown on solid DM agar in the absence of antibiotics for 5–7 days at 28 °C to reach 24 generations (calculated from the number of divided cells). Subsequently, the culture was diluted and plated on DM agar in the presence or absence of tetracycline. The number of colonies grown in each plate was counted, and colonies of 50 or 100 were picked up from plates lacking antibiotic, then streaked on plates containing antibiotics to further confirm the result. The average ratio of plasmid loss per generation was calculated from these data.

2.4. DNA sequence analysis

DNA sequence determination was performed on an Applied Biosystems 373A automated DNA sequencer (Applied Biosystem). The sequence is available as GenBank Accession No. AY380839. Searches of the GenBank database were performed with the FASTA [22] and BLASTN [23] programs. Sequence alignments were performed by the Clustal method of the MEGALIGN program of the DNASTAR software package.

3. Results and discussion

3.1. Determination of the replicon of pCXC100

The primary restriction map of pCXC100 was generated in this study by restriction of the high-purity pCXC100 DNA isolated from the wild-type *L. xyli* subsp. *cynodontis* (Fig. 1). The location of the plasmid replicon was then determined. High-purity pCXC100 DNA was then digested with restriction enzymes that generated DNA fragments covering the full-length pCXC100 with 3–8 kb overlaps between adjacent DNA fragments. These fragments were cloned into pBR325, resulting in the recombinant plasmids pLXC101 to pLXC105 (Fig. 1) that were transformed into a plasmid-free *L. xyli* subsp. *cynodontis* isolate. Because the ColE1-based plasmids could not yield *L. xyli* subsp. *cynodontis* transformant [19], we can conclude that any resulting viable clones must host the recombinant plasmid containing the replicon of pCXC100. We found that pLXC101 and pLXC105 yielded viable and stable *L. xyli* subsp. *cynodontis* transformants. Thus the replicon of pCXC100, responsible for both the plasmid replication and stability, is located within the overlap of pCXC100 sequence inserted in pLXC101 and pLXC105, which was the 6.5 kb *Nco*I–*Bgl*II fragment (Fig. 1).

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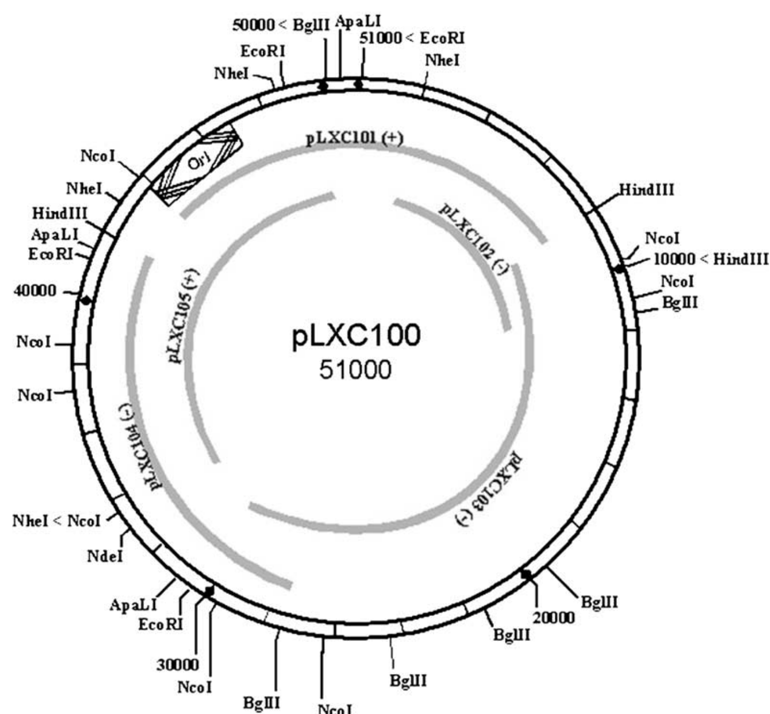

Fig. 1. Physical and restriction maps of pCXC100. The 51 kb plasmid is shown as a circular molecule, with nucleotide number 1 starting from an *Eco*RI site. The location of restriction sites for *Apa*L, *Bgl*II, *Eco*RI, *Hin*dIII, *Nco*I, *Nhe*I and *Nde*I are indicated. A "+" indicates that viable *Lxc* transformants were obtained when the indicated DNA fragment was insert into pBR325 and a "−" indicates that no viable transformants were obtained. The minimal replication origin is shown as checkerboard.

A series of subclone plasmids (pLXC106–pLXC111) were then constructed to further locate the replicon of pCXC100 (Fig. 2). It was shown that all plasmids containing the 3.0 kb *Nco*I–*Ava*I DNA fragment yielded viable transformants, while those lacking this DNA region or containing only a part of this region did not (Fig. 2). Note that plasmid preparation from *L. xyli* subsp. *cynodontis* cells was readily restriction digested in vitro, suggesting that no DNA modification occurs in this organism to maintain the plasmid integrity. We can conclude that the untransformed plasmids must be due to the loss of replication ability, not the restriction barrier that limits transformation. On the other hand, plasmid containing the 3.0 kb *Nco*I–*Ava*I DNA fragment *alone* had a poor stability, demonstrated by the nearly 14% plasmid-loss per generation. Extending the DNA presence about 1 kb downstream from the *Ava*I site to the adjacent *Xho*I site decreased the plasmid loss to 1% per generation. Full plasmid stability was ensured by the presence of one more kilobase DNA from the *Xho*I site to the adjacent *Eco*RI site (Fig. 2). Therefore, we concluded that the replication function of pCXC100 was located within the 3.0 kb *Nco*I–*Ava*I DNA region; the major stability function was encoded by the 1.0 kb *Ava*I–*Xho*I DNA region, and the additional 1.0 kb *Xho*I–*Eco*RI DNA region may encode additional stability function.

3.2. Organization of the replicon of pCXC100

The nucleotide sequence of the 5.0 kb replicon, *Nco*I–*Eco*RI DNA fragment encoding the replication and stability functions, was then determined (Accession No. in GenBank, AY 380839). The identified replicon contains 4992 nucleotides with an overall G + C content of 65%. The high G + C content of the replicon sequence is

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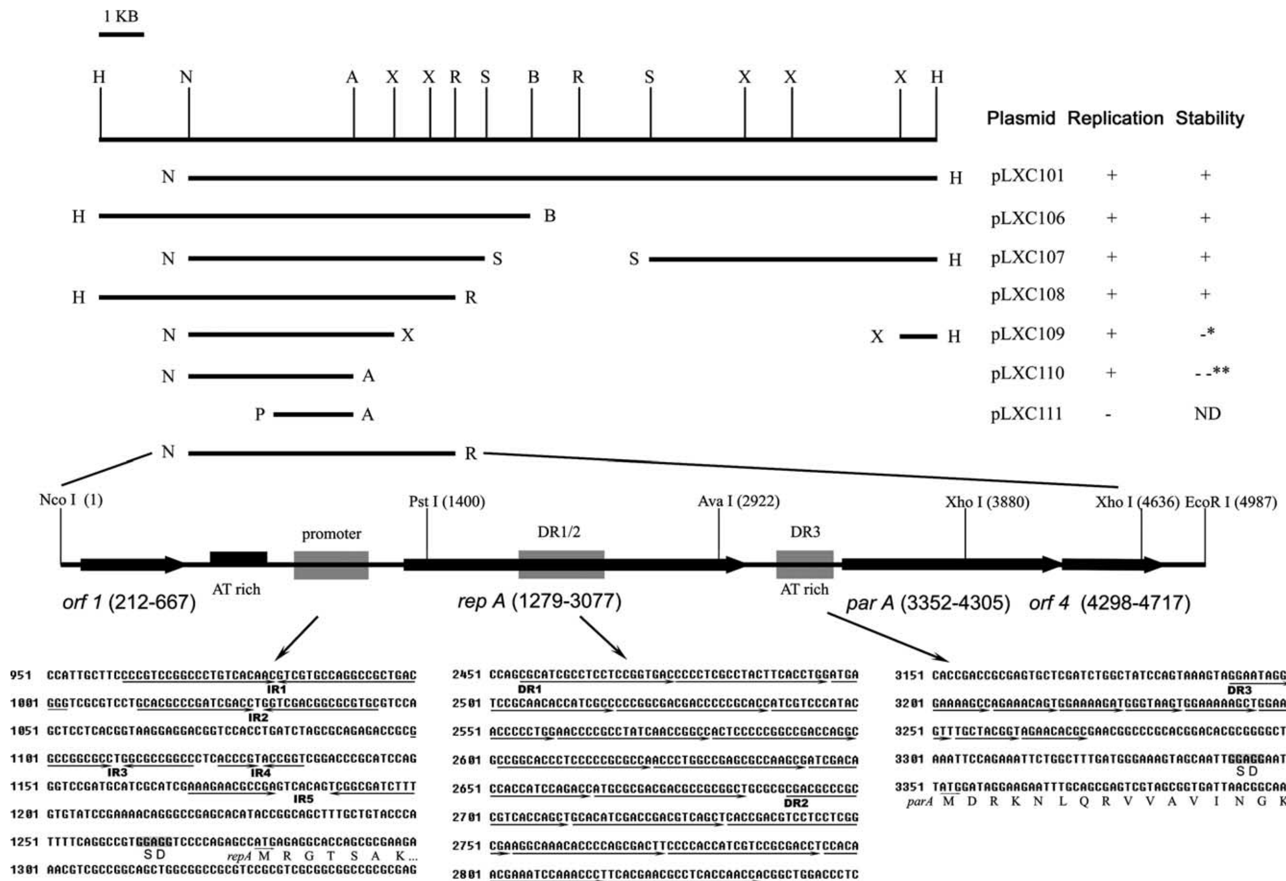

Fig. 2. The location and organization of the pCXC100 replicon. The 17 kb *Hin*dIII fragment of pCXC100 was drawn to scale with the corresponding restriction sites indicated, A – *Ava*I, B – *Bgl*II, H – *Hin*dIII, N – *Nco*I, P – *Pst*I, R – *Eco*RI, S – *Sma*I, X – *Xho*I. All the plasmids listed were generated as described in Table 1. The restricted DNA fragments of pCXC100 cloned into pBR325 (pLXC101, 106–111) were indicated. The restriction enzymes used to generate each DNA fragment are listed at their ends. The ability of each plasmid to yield viable *L. xyli* subsp. *cynodontis* transformants and the stability in the bacterium are indicated on the right side. A "+" indicates the plasmid was capable of yielding viable clones or was stable when propagating in *L. xyli* subsp. *cynodontis*, and a "–" indicates the plasmid was incapable of yielding viable clones or was not stable when propagating in the bacterium. One asterisk indicates the plasmid loss at 1% per generation, and the double-asterisk indicates the plasmid loss at 14% per generation. The organization of the encoded proteins and cis-acting elements in the 4992-nt replicon was shown at the lower panel. The sequence of several *cis*-acting elements is listed at the bottom. IR indicates the invert repeat sequence, and DR indicates the direct repeat sequence.

similar to that of the ribosomal RNA gene [24]. Proteins potentially encoded in the replicon were identified by searching the database. The search criteria were set to have the open reading frame (ORF) consist of at least 50 codons preceded by a potential Shine–Dalgarno sequence at an appropriate distance (6–15 bp) from one of the commonly used initiation codons (AUG, UUG, and GUG). Shine–Dalgarno sequence was determined by complementation with the 3′ sequence of 16S rRNA of *L. xyli* subsp. *cynodontis* (5′ GGCUGGAUCACCUCC-UUUCU 3′) [24]. Search results revealed the presence of a putative replication protein in the *Nco*I–*Ava*I DNA region and two putative stability proteins in the *Ava*I–*Eco*RI region, which is consistent with the experimental results described above. These putative ORFs, together with a number of putative *cis*-acting elements, depict the primary structure of the plasmid survival kit of pCXC100 plasmid (Fig. 2).

3.2.1. The large replication protein RepA and regulatory elements

Consistent with our finding that *Nco*I–*Ava*I region was responsible for the replication of plasmids in *L. xyli* subsp. *cynodontis*, the largest ORF in this region encodes an arginine-rich protein that shares homology with the pLR7 family of replication proteins, and was therefore designated RepA (Fig. 3) [25–29]. We noticed that the sequence of the putative RepA is much longer than those of the pLR7 family, with the extra sequence being dispersed between the conserved regions. Nevertheless, the putative RepA of pCXC100 shares the most conserved regions the Rep proteins in the pLR7 family [27]. The conservation at the N-terminal region is much more extensive than that at the C-terminal (Fig. 3 and data not shown). Like the replicon of the plasmid pLR7 [25], a number of invert repeats are located in the putative promoter region of RepA (Fig. 2). These invert repeats may serve as *cis*-acting elements to regulate the RepA expressions. The putative RepA also contains three helix-turn-helix DNA-binding motifs (184–205 aa, 195–216 aa, 337–358 aa) that are the characteristic structure of Rep proteins [2]. Deletion of the N-terminus and the putative promoter region of RepA resulted in the loss of plasmid replication ability in *L. xyli* subsp. *cynodontis*, but partial deletion of C-terminus had no effect on the plasmid replication (see pLXC110 and pLXC111 in Fig. 2), confirming the importance of the promoter and N-terminal region. On the other hand, the 51 aa in the C-terminal region of RepA may be not essential in plasmid replication, as demonstrated by the replication capability of the deletion mutant pLXC100.

A conserved DNA region upstream of the *rep* gene of the plasmid was found in the pLR7 related plasmids [27,28], however, no such homology was found upstream of the *repA* gene of pCXC100. Nonetheless, two 21-nt repeat sequences, repeated 11 and 7 times separately, were evident within the deduced sequence of RepA (DR1 located in 2455–2685 bp, DR2 located in 2691–2837 bp Fig. 2). These repeat sequences may represent a potential regulation site that is similar to the 54 bp iteron within the deduced sequence of RepA of the plasmid pCI2000 from gram-positive bacterium *Lactococcus lactis* [9]. An

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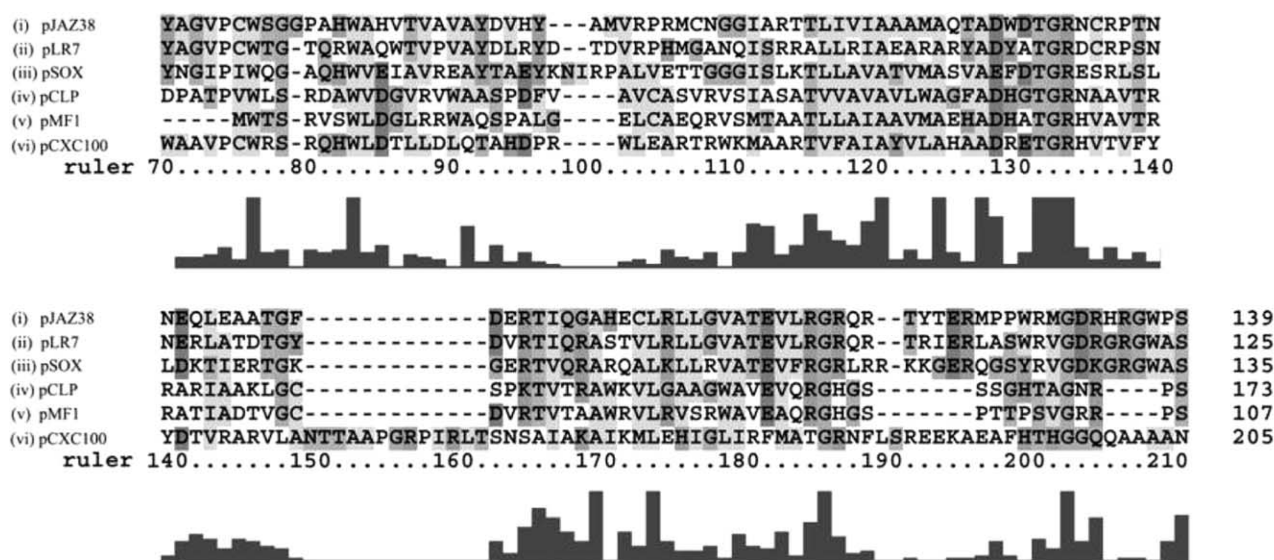

Fig. 3. Comparison of the amino acid sequence of the N-terminal region of pCXC100 RepA with a number of homologous *rep* proteins encoded by different plasmids: (i) pJAZ38 from *Mycobacterium fortuitum* (Accession No. AAB62814), (ii) pLR7 from *Mycobacterium avium* (Accession No. AAA82043), (iii) pSOX from *Rhodococcus* sp. strain X309 (Accession No. AAC73055), (iv) pCLP from *Mycobacterium celatum* (Accession No. AAD42965), (v) pMF1 from *Mycobacterium fortuitum* (Accession No. CAB43095), (vi) pCXC100 from *Lonfsonia xily* subsp. *cynodontis* (Accession No. AY380839). The amplitude of the gray bar below each amino acid position indicates the sequence similarity among all the list homologous proteins from different plasmids.

AT-rich region was located before the putative promoter of RepA (Fig. 2), representing another potential regulation site for the plasmid replication [2,29]. Upstream of the AT-rich region is the ORF1 that potentially encodes a protein consisting 152 amino acids. A search of the database showed that a large portion of the ORF1 protein is homologous to the putative type I restriction-modification system methylase of the *Corynebacterium efficiens* (gi:25028882). It is not clear if ORF1 encodes any function related to the methylation of plasmid DNA.

3.2.2. *The partition protein ParA and* par *locus*

Two ORFs were identified a few hundred nucleotides downstream of the *repA gene* and in the *Ava*I–*Eco*RI region encoding the plasmid stability function. The putative protein encoded by the upstream ORF (317 amino acids) is significantly homologous to the ParA family of Walker-type ATPases that are involved in active partition, and was thus designated ParA [3] (Fig. 4). This newly identified ParA contains two highly conserved ATP-binding motifs (motifs I and III) and two other conserved motifs (motifs II and IV) (Fig. 4) that characterize the ParA family of ATPase [9,30–32]. The presence of N-terminal 177 amino acids comprising all four conserved motifs dramatically increased the plasmid stability (Fig. 2), indicating that the conserved region of *parA* gene plays major roles in plasmid stability.

There are nine purine-rich direct repeat sequences (DR3) located within the putative promoter region of *parA* (Fig. 2). DR3 might be the *cis*-acting centromere-like site, namely *parS* [3].

ORF4 (encoding 139 amino acids) was located downstream of *parA* gene with a little overlap. No homologous protein was found in the protein database. As it was demonstrated above, the region containing both the C-terminal region of *parA* gene and ORF4 further stabilized the plasmid, extending the plasmid stability from 99% to 100% in our analysis (Fig. 2). However, it is unclear which resultant protein is responsible for conferring this increased stability.

Almost all known plasmid-encoded *par* loci consist of three components: a *cis*-acting centromere-like site (*parS*) and two trans-acting proteins (ParA and ParB) forming a partition complex at *parS*. The upstream gene generally encodes an ATPase and the downstream gene encodes a protein binding to *parS* [3,32]. It is known that type I *par* loci contain the Walker-type ATPase and the type II contains ATPase that belongs to the actin/hsp70 superfamily. Two subgroups of type I partition loci were discovered. Type Ia loci encode large ParA (251–420 aa) and ParB (182–336 aa) homologues, and the *parS* site is located downstream of *parB*. In contrast, the type Ib loci encode smaller ParA (182–336 aa) and ParB (46–113 aa) proteins, and the *parS* site is located in the promoter region upstream of the *parAB* operon [3]. The sizes of the ParA and the Orf4 protein, and the location of the potential *parS* site (DR3) suggest that the *par* locus of pCXC100 replicon belongs to the type Ib loci, in which the ORF4 should encode ParB protein. This is similar to the *par* locus of plamids pAW63 and pCI2000 isolated from gram-positive bacteria *Bacillus thuringiensis* and *Lactococcus lactis*, respectively [3,9,11]. However, a motif search of the Orf4 protein found no typical helix-turn-helix DNA-binding motif, weakening the possibility that the Orf4 protein serves as a DNA binding protein to regulate the expression and activity of ParA protein [3]. The detail function of ORF4 remains to be elicited.

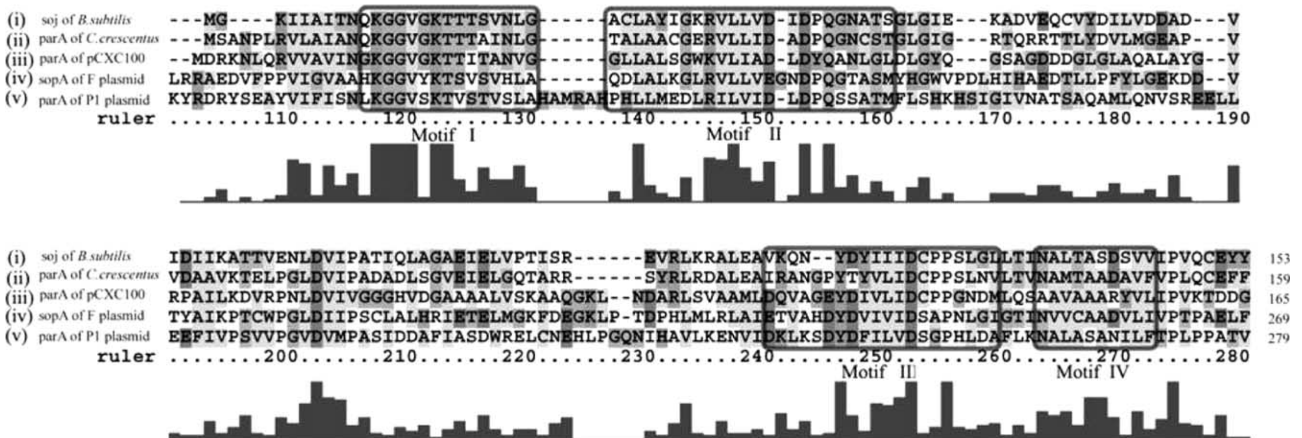

Fig. 4. Comparison of the amino acid sequence of the N-terminal region of pCXC100 ParA with a number of homologous proteins in the databases: (i) Soj chromosomal protein from *Bacillus subtilis* (Accession No. P37522), (ii) ParA chromosomal protein from *Caulobacter crescentus* (Accession No. U87804), (iii) ParA pCXC100 (Accession No. AY380839), (iv) SopA of the F plasmid (Accession No. P08866), (v) ParA of the P1 plasmid of *E. coli* (Accession No. P07620). The framed boxes marked Motif I and Motif III represent the ATPase motif region I and region II. The framed boxes designated Motif II and Motif IV represent the additional motifs considered to be involved in protein–protein interactions or interaction with the cell membrane. The amplitude of the gray bar below each amino acid position indicates the sequence similarity among all the listed homologous proteins.

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