JOURNAL OF BACTERIOLOGY, Sept. 1987, p. 3952–3962
0021-9193/87/093952-11$02.00/0

Vol. 169, No. 9

Structural and Genetic Analyses of a *par* Locus That Regulates Plasmid Partition in *Bacillus subtilis*

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Received 13 February 1987/Accepted 3 June 1987

The *Bacillus* plasmid pLS11 partitions faithfully during cell division. Using a partition-deficient plasmid vector, we randomly cloned DNA fragments of plasmid pLS11 and identified the locus that regulates plasmid partition (*par*) by *cis* complementation in *Bacillus subtilis*. The cloned *par* gene conferred upon the vector plasmid a high degree of segregational stability. The *par* locus was mapped to a 167-base-pair segment on pLS11, and its nucleotide sequence was determined. The cloned *par* fragment regulated the partition of several different *Bacillus* replicons, and it only functioned in *cis*; it did not contain the replication function nor elevate the plasmid copy number in *B. subtilis*. The expression of *par* was orientation specific with respect to the replication origin on the same plasmid. We propose that the pLS11-derived *par* functions as a single-stranded site that interacts with other components involved in plasmid partition during cell division.

Many bacterial plasmids partition faithfully into daughter cells during cell division. The high fidelity of partition exhibited by unit-copy plasmids such as F and P1 suggests that plasmids do not segregate randomly. The P1 plasmid uses a site-specific recombination system (*loxP-cre*) to resolve replicon dimers for proper plasmid partition (5). In addition, P1 and F plasmids carry partition genes encoding *trans*-acting protein products as well as *cis*-acting sites (2–5, 22). On the other hand, multicopy plasmids, in theory, can be efficiently maintained in a growing bacterial population if they simply segregate randomly during cell division. However, data on the multicopy *Escherichia coli* plasmids CloDF13 (33) and pSC101 (6, 20, 21) suggest that specific mechanisms are used to control the partitioning of this type of plasmid.

Several genetic loci can affect plasmid maintenance, including those involved in plasmid DNA replication, incompatibility, and genetic recombination. Recently the genetic elements termed *par*, which control plasmid partitioning, were identified and characterized genetically and physically. For example, the *cis*-acting *par* element from the *E. coli* plasmid pSC101 was cloned molecularly and its complete nucleotide sequence was determined (20, 21). The pSC101 *par* locus does not strictly specify plasmid incompatibility and does not appear to encode protein products. The *par* loci of P1 and mini-F plasmids consist of *cis*-acting sites as well as genes that specify protein products participating in plasmid partition (3, 4, 22). Thus there exist several different systems to control plasmid partition. Those derived from large replicons, such as P1 and F, involve multiple partition genes; they might also be involved in plasmid incompatibility. Those derived from relatively small replicons, such as pSC101, probably function as the sites that interact with other cellular components during plasmid partition. The precise underlying mechanisms that govern plasmid partition are not yet completely understood, and they may vary among different plasmids.

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Many naturally occurring plasmids, irrespective of their copy number, are stably maintained in a growing bacterial population. Chimeric plasmids generated by in vitro recombination methods, however, are frequently partition deficient because they usually consist of only the replication origin, the associated replication functions, and one or two additional selectable markers such as antibiotic resistance genes. We have previously constructed a *Bacillus* vector, pOG1196 (9), from the *Staphylococcus aureus* plasmids pC194 and pUB110 (11, 14). Plasmid pOG1196 and its bifunctional derivative pOG2326 are partition deficient (Par^-) in *B. subtilis*. Using an enrichment procedure, we identified a cloned partition (*par*) element from the cryptic *Bacillus* plasmid pLS11 (28), which complemented the Par^- pOG2326 vector. The molecular cloning and some of the properties of this *par* locus are described here.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *B. subtilis* BD224 (*trpC2 thr-5 recE4*) (11) and *E. coli* CS412 (9) were used as the hosts for molecular cloning. Plasmids pBR322 (7), pC194 (14), pUB110 (11), pOG2326 (9) pLS11 (28; reported as pTA1060 in reference 32), pBD15 (pE194 *cop-6*) (12), pJH101 (8), and pLP1201 (23) have been described previously. The broth medium used was 2× LB (16). The solid medium was prepared from Penassay agar (Difco Laboratories). For culturing *E. coli*, the medium was supplemented with thiamine hydrochloride to 5 μg/ml. When needed, chloramphenicol, erythromycin, and kanamycin were each used at a final concentration of 5 μg/ml, ampicillin was used at 50 μg/ml, and tetracycline was used at 15 μg/ml.

Measurement of partition phenotype. Plasmid-bearing bacteria were initially grown in the presence of the appropriate antibiotic. An antibiotic-free broth medium was then inoculated either with a single colony of the testing strain picked from the plate or with washed cells from a broth culture containing antibiotic. These cultures were allowed to reach the mid- or late-exponential phase of growth at 37°C with shaking; more than 5×10^5 cells of the cultures were then

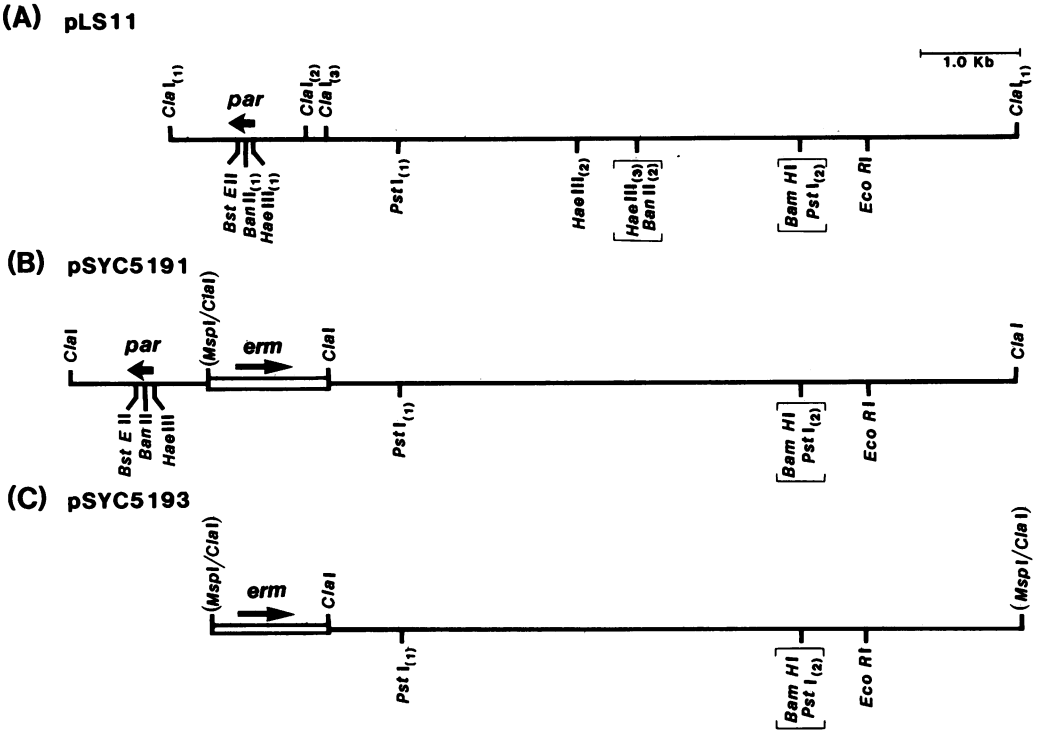

FIG. 1. Linear physical maps of plasmid pLS11 and its derivatives.

transferred to inoculate another fresh culture of the same medium. This serial propagation was carried out until the desired generation number was reached.

Bacteria from the cultures were sampled by being plated on antibiotic-free plates as well as on plates containing the appropriate antibiotic to select for the plasmid-bearing cells. The fraction of plasmid-bearing cells in the population was then calculated. If more than 50% of the population were plasmid-free cells, then individual colonies from antibiotic-free plates were further tested on antibiotic-containing plates for plasmid-specified resistance to determine the fraction of plasmid-bearing cells.

To ensure greater accuracy, the sample size of the plasmid-bearing population used for data collection was always more than 100. We scored the plasmid phenotype Par^+ if more than 99% of the tested cells contained plasmid after a minimum of 30 generations of growth in the absence of antibiotic selection. The Par^- plasmids described here showed a high rate of segregation; usually less than 50% of cells contained plasmid after 30 generations of growth without selection.

DNA manipulations. Preparation of plasmid DNA by the cleared-lysate and CsCl gradient centrifugation methods, manipulations of DNA fragments with DNA polymerase and T4 DNA ligase, electrophoretic analysis of DNA in agarose and polyacrylamide gels, and transformation of *E. coli* cells were essentially as described previously (17). All enzymes were purchased from commercial suppliers except T4 DNA ligase, which was kindly provided by D. Gelfand, Cetus Corp. The chemical cleavage method (18) and the dideoxynucleotide chain termination method (24) were used to determine the DNA sequence of *par* on both strands. A 24-nucleotide synthetic fragment, TGATGCGATTAAGCT TGGCGTGAG, was used to modify the cloned *par* sequence by the method of Zoller and Smith (35).

B. subtilis competent cells was prepared by the method of Anagnostopoulos and Spizizen (1) with minor modifications.

Plasmid copy number determination. The method for determining plasmid copy number was described by Gryczan et al. (12).

RESULTS

Plasmid pLS11 is partition proficient. The cryptic plasmid pLS11 was originally isolated from *B. subtilis* IFO3022 by Tanaka et al. (28). It is an 8.4-kilobase (kb) plasmid with an estimated copy number of 7. This plasmid has also been reported (designated as pTA1060) by Uozumi et al. (32). However, in a more recent report (34), this IFO3022 strain was classified as *B. amyloliquefaciens*. The host range of this plasmid probably extends into both species, since a homologous *B. amyloliquefaciens* plasmid, pFTB14, replicates in both *Bacillus* species (34). We were unaware of any selection pressure that selects for the presence of this plasmid in the bacterial population, and we supposed that pLS11 contained within its genome a genetic element(s) that controls plasmid partition.

To better analyze the partition property of pLS11, we inserted into it an antibiotic resistance marker. The *ermC* gene encoding erythromycin resistance (Em^r) was excised from pBD15 by digestion with *Msp*I and *Cla*I. This 1.3-kb fragment was ligated to pLS11 DNA that had been partially digested with *Cla*I, and Em^r BD224 transformants were selected. From several clones characterized, we identified two plasmids that had the *ermC* gene fragment replacing either the smallest (0.21-kb) *Cla*I fragment of pLS11 or the two smaller (0.21- and 1.4-kb) *Cla*I fragments, giving rise to plasmids pSYC5191 and pSYC5193, respectively. The physical maps of pLS11 and its Em^r derivatives, pSYC5191 and pSYC5193, are shown in Fig. 1.

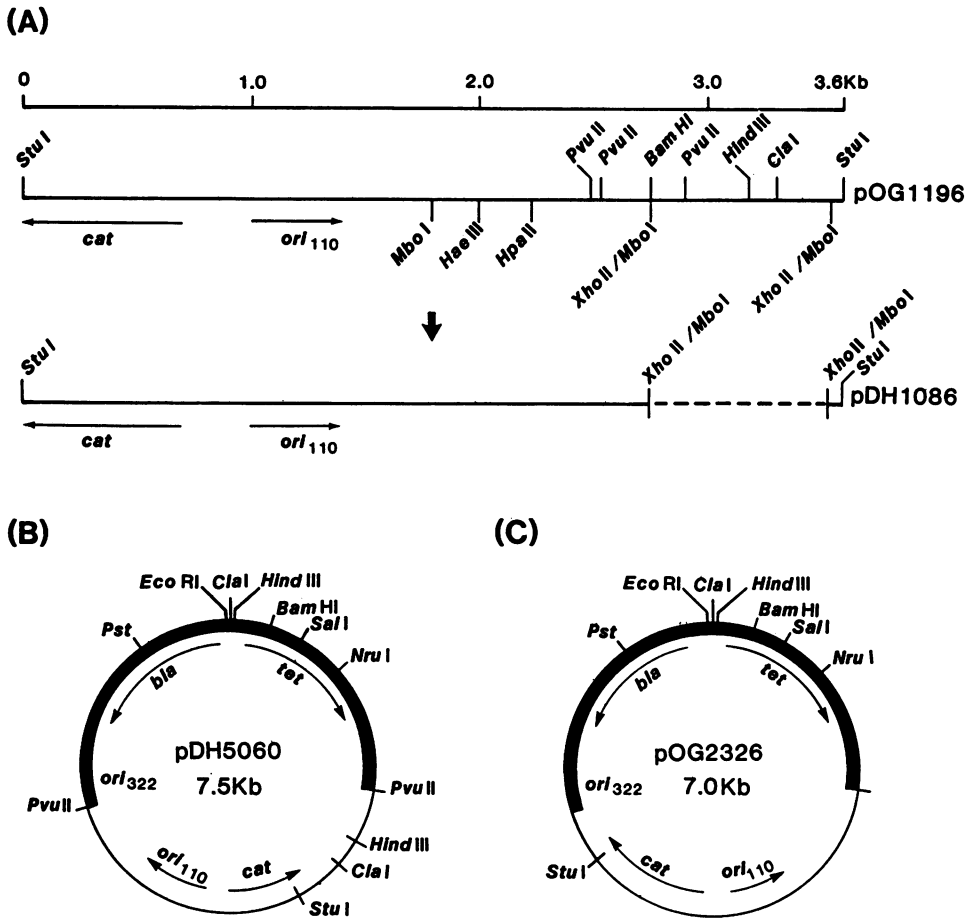

FIG. 2. Physical maps of plasmid pOG1196 and its derivatives. (A) Plasmids pOG1196 and pDH1086, shown as linearized at the unique *Stu*I site in the *cat* gene. (B) Plasmid pDH5060. (C) Plasmid pOG2326. ▬, Sequences derived from *E. coli* plasmid pBR322. The locations of the pUB110-derived (ori_{110}) and the pBR322-derived (ori_{322}) replication origins, as well as the β-lactamase (*bla*), tetracycline resistance (*tet*), and *cat* genes are shown. We observed two closely clustered *Pvu*II sites in the pUB110-derived region on pOG1196 (9). The reported pUB110 sequence contained a single *Pvu*II site at the same location (19). The discrepancy is not clear.

We tested the partition phenotype of these two Em^r plasmids by the procedures described in Materials and Methods. After 50 generations of growth, all the cells in the BD224(pSYC5191) culture were Em^r, whereas only 7% of the cells in the BD224(pSYC5193) culture retained the plasmid. These data indicate the following. (i) The large *Cla*I fragment of pLS11 contains the gene(s) for plasmid replication. (ii) The 1.4-kb *Cla*I fragment contains the genetic element that is required for faithful plasmid partition, but is not essential for plasmid replication. (iii) Plasmid pLS11 is partition proficient (Par^+), and deletion of the 0.21-kb *Cla*I fragment does not affect plasmid partition and plasmid replication.

Plasmid pOG1196 and its derivatives are partition deficient in *B. subtilis*. The chimeric plasmid pOG1196 (9) is composed of the replication origin (ori_{110}) fragment from plasmid pUB110 and the chloramphenicol acetyltransferase (*cat*) gene of pC194; both parental plasmids are of *S. aureus* origin. Shuttle vectors pDH5060 and pOG2326 were constructed between the *E. coli* plasmid pBR322 and the *Bacillus* replicon pOG1196, or a derivative of pOG1196 (Fig. 2). Plasmid pDH5060 is generated by cloning the large *Pvu*II fragment of pOG1196 into the *Pvu*II site of pBR322. This plasmid replicates in both *E. coli* and *B. subtilis* and has been used to construct *B. subtilis* genomic libraries (23). A deletion derivative (pDH1086; Fig. 2) with the smallest *Mbo*I fragment removed was constructed. The large *Pvu*II fragment of pDH1086 was cloned into pBR322 at the *Pvu*II site to generate the bifunctional plasmid pOG2326. No *Pvu*II sites were regenerated in pOG2326, probably as a result of contaminating nucleases in the enzyme preparation used in this experiment.

The partition properties of these pOG1196-derived shuttle vectors in BD224 cells were determined (Fig. 3). Plasmid pOG2326 was very unstable. A large fraction of cells grown under selection pressure, i.e., those freshly harvested from chloramphenicol-containing medium or from colonies on chloramphenicol-containing plates, were already "plasmid free." The methods used did not directly measure the presence of plasmids in each cell. Cells that did not contain plasmid, as well as those that did not contain sufficient copies of plasmid to allow plating on chloramphenicol-containing plates, were scored as plasmid free. Those that remained chloramphenicol resistant were derived from cells containing at least one, and possibly several, copies of plasmids. While both pOG2326 and pDH5060 segregated at a high rate, pDH5060 was more stable than pOG2326. This higher level of stability of pDH5060 over pOG2326 may be due to the additional sequence present in the pOG1196-derived portion in pDH5060 (Fig. 2).

Molecular cloning of the *par* locus from pLS11. Genetic complementation of the partition deficiency of pOG2326 by molecular cloning is one method to directly identify the *par* locus from pLS11. Plasmid DNA of pLS11 was isolated from strain IFO3022 and digested to completion with *Mbo*I. This yielded about 20 fragments. They included 3 fragments ranging between 1.0 and 1.3 kb, 7 fragments between 0.3 and 1.0 kb, and about 10 fragments smaller than 0.3 kb. These *Mbo*I fragments were ligated to *Bam*HI-digested pOG2326 DNA and then transformed into *E. coli* CS412. Plasmid DNA, prepared from a pool of about 100 ampicillin-resistant (Ap^r), tetracycline-sensitive (Tc^s) *E. coli* clones, was used to transform *B. subtilis* BD224 cells. About 1,000 Cm^r *Bacillus* transformants were scraped off the plates and inoculated into a single broth culture without chloramphenicol selection. This culture was propagated for about 50 generations by serial dilutions, and partition-deficient plasmids were allowed to segregate. Cells from this culture were then grown overnight in chloramphenicol-containing broth to enrich for the surviving clones that potentially carried partition-proficient chimeric plasmids. After two cycles of this type of segregation-enrichment treatment, eight individual clones were randomly chosen for further characterization.

Plasmids from these Par^+ clones were isolated and shown to be approximately the same size. As it was subsequently determined that the insert from the representative plasmid, designated as pOG2381, was likely to have resulted from the ligation of two independent *Mbo*I fragments, the eight clones obtained are probably siblings. The data on the stability analysis of pOG2381 are shown in Fig. 3.

Mapping of the cloned *par* locus in pOG2381. Plasmid pOG2381 and the parent plasmid pOG2326 were each digested with *Sau*3A (an isoschizomer of *Mbo*I) to completion, and the fragment patterns were compared by agarose gel electrophoresis. The only difference observed was the presence of an extra 1.2-kb fragment in the pOG2381 digest (data not shown). This suggested that the 1.2-kb insert in pOG2381 was responsible for the Par^+ phenotype. The physical map of pOG2381 is shown in Fig. 4. To rule out the possibility that the Par^+ phenotype was caused by an unanticipated mutation(s) in the host genome or in the pOG2326 vector sequence, we retransformed pOG2381 into competent BD224 cells. Once again, the Par^+ phenotype was observed in these transformants. This indicates that the Par^+ property is a plasmid-associated phenotype. In addition, we purified the 1.2-kb insert from pOG2381 and cloned it into the *Bam*HI site in plasmid pOG2326. Recombinant plasmids identical to pOG2381 were obtained, and they had the same partition phenotype as pOG2381. Thus the genetic information governing partition proficiency resides within this 1.2-kb *Mbo*I (*Sau*3A) fragment.

During reconstruction of pOG2381 by molecular cloning of the purified 1.2-kb *par* fragment into pOG2326, we also obtained plasmids carrying the *par* fragment in the opposite

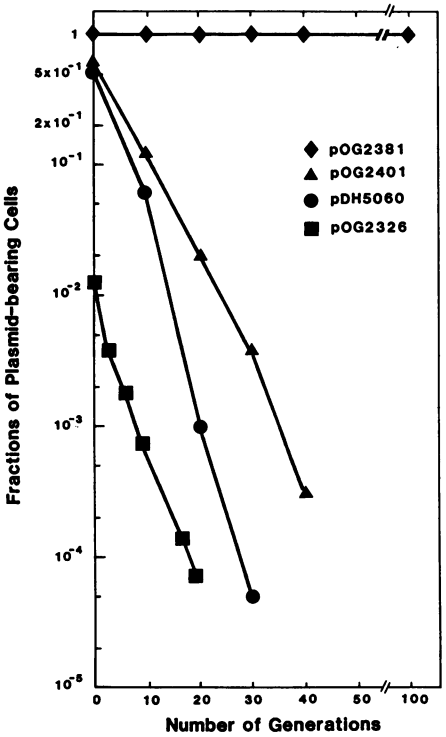

FIG. 3. Rate of plasmid loss in *B. subtilis* BD224 in the absence of chloramphenicol selection. Cells of each tested strain were initially grown in the presence of chloramphenicol and used to inoculate a fresh broth medium without selection at zero generation time. The fraction of Cm^r cells in the cultures was determined at various stages of culture propagation.

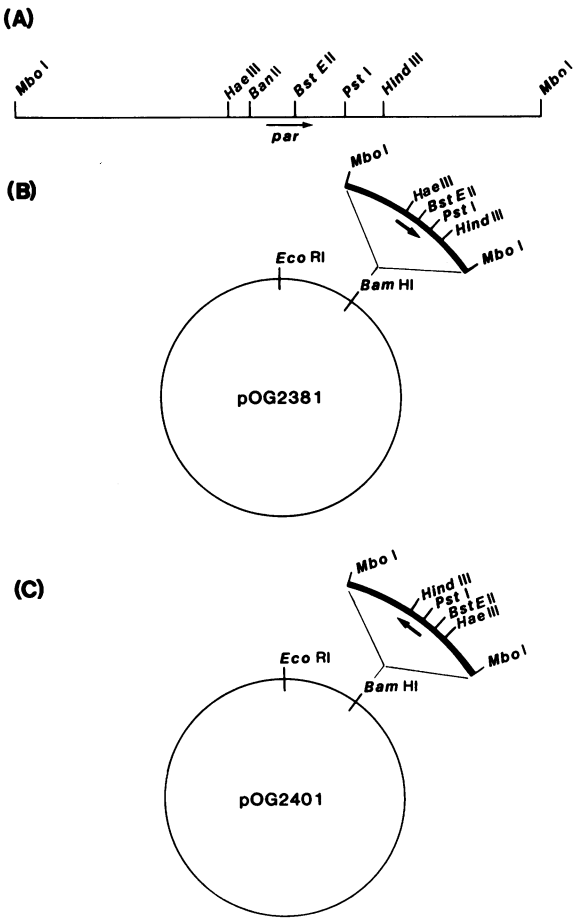

FIG. 4. Physical maps of the *par*-containing plasmids and the cloned *par* fragment. The 1.2-kb *Mbo*I fragment (A) was inserted at the *Bam*HI site of plasmid pOG2326, to generate plasmids pOG2381 (B) and pOG2401 (C). The orientation of *par* (shown by the arrow) is arbitrarily assigned as described in Results. The pBR322-derived replication origin is in the same orientation (not shown) as that of ori_{110}.

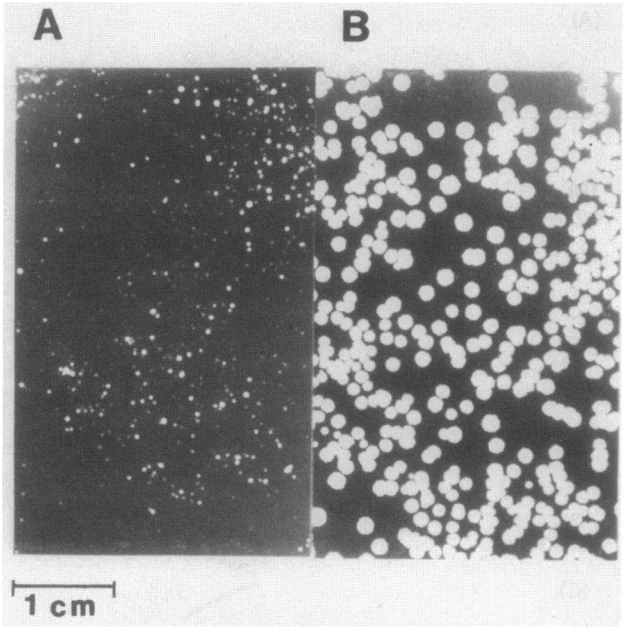

FIG. 5. Correlation of plasmid partition phenotype and colony size. BD224 cells harboring Par$^-$ plasmid pOG2401 (A) and Par$^+$ plasmid pOG2381 (B) were plated on chloramphenicol-containing plates. The pictures were taken after 20 h of incubation at 37°C.

orientation. The physical maps of plasmid pOG2381 and a representative plasmid (pOG2401) that has the *par* fragment inserted in the opposite orientation are shown in Fig. 4. Unexpectedly, plasmid pOG2401 was found to be Par$^-$. The rates of segregation for plasmids pOG2401, pOG2381, and pOG2326 were compared (Fig. 3). The simplest interpretation of this orientation effect is that the expression of the cloned *par* locus requires transcription from a promoter in the vector that reads across one of the junctions into the insert to activate the *par* locus. However, our subsequent data do not support this hypothesis (see below).

Plasmids pOG2401 and pOG2381 exhibit Par$^-$ and Par$^+$ phenotypes, respectively, in *B. subtilis* BD224. Their respective partition phenotypes are also reflected in their colony size on chloramphenicol-containing plates after overnight incubation at 37°C; BD224(pOG2401) cells formed small colonies, whereas BD224(pOG2381) cells gave rise to larger colonies that were more uniform in size (Fig. 5). This provided a convenient visual screening for the Par phenotype. This difference was not totally unexpected, because of the extremely high rate of segregation of pOG2401 and pOG2326 in BD224 cells, in contrast to the Par$^+$ plasmid pOG2381 (Fig. 3).

The Par$^+$ plasmid pOG2381 was also tested for its partition phenotype in *E. coli* CS412. The segregation rate was similar to that of the parent pOG2326 plasmid, and both were found to be Par$^-$ (data not shown). Hence we concluded that the pLS11-derived *par* sequence does not function in *E. coli*.

Mapping of the *par* locus on pLS11. Our mapping data indicated that the clustered *Bst*EII and the *Hae*III sites were located adjacent to the *Pst*I site in the cloned *par* fragment in pOG2381 (Fig. 4). However, we did not observe a similar linkage of these sites in pLS11 from restriction mapping experiments, and we detected the common 0.15-kb *Hae*III-*Bst*EII fragment only in pLS11 and pOG2381. This *Hae*III-*Bst*EII fragment mapped in the 1.4-kb *Cla*I fragment of pLS11 (Fig. 1). Using the *Hae*III-*Bst*EII fragment derived from pOG2381 as a probe for Southern blotting (26), we also confirmed that its sequence is homologous to that present in the 1.4-kb *Cla*I fragment of pLS11. The most plausible explanation for this discrepancy is that during the shotgun cloning process that generated plasmid pOG2381, two or more *Mbo*I fragments were ligated and inserted into the *Bam*HI site of pOG2326. Subsequently, an in vivo deletion event took place that removed the internal *Mbo*I sites within the tandem inserts. This suggested that we should be able to identify a *par*-containing *Mbo*I fragment from pLS11 that is different from the 1.2-kb *Mbo*I fragment in pOG2381. Indeed, we found a 0.71-kb *Mbo*I fragment located within the 1.4-kb *Cla*I fragment from pLS11 (data not shown); this 0.71-kb *Mbo*I fragment contained the *par* locus.

We cloned the 0.71-kb *Mbo*I fragment into the *Bam*HI site of pOG2326 and generated plasmids pSYC1122 and pSYC1123. The orientation of the common *Hae*III-*Bst*EII fragment in pSYC1122 was the same as that in pOG2341, and both plasmids were Par$^-$. Similarly, pSYC1123 was Par$^+$, and it had the 0.71-kb *par* fragment inserted in the same orientation as in pOG2381. This confirmed the earlier observation of the orientation dependence of *par*. It also indicates that the *par* cloned in pOG2381 is likely to be functionally intact.

Mapping of the *par* locus by subcloning. Within the 1.2-kb *Mbo*I *par* fragment, there is a *Hae*III site located ca. 0.35 kb from the *Hin*dIII site (Fig. 4). We mapped the *par* locus between the *Hae*III and the *Hin*dIII sites. We inserted the purified 0.35-kb *Hae*III-*Hin*dIII fragment between the repaired *Eco*RI site and the *Hin*dIII site in pOG2326 and transformed the resulting plasmid (pSYC676; Fig. 6A) into *B. subtilis* BD224. BD224(pSYC676) transformants were phenotypically Par$^+$.

The same method was used to clone this 0.35-kb *Hae*III-*Hin*dIII fragment between the repaired *Bam*HI site and the *Hin*dIII site of pOG2326, and it generated plasmid pSYC677 (Fig. 6B). However, pSYC677 was tested to be Par$^-$. Because both pSYC677 and pOG2401 contained the cloned *par* sequence and yet both were phenotypically Par$^-$, these results indicated that the mere presence of the *par* fragment in the *cis* configuration is not sufficient to confer the Par$^+$ phenotype upon the vector plasmid. The orientation of *par* in these plasmids appears to be an important parameter in *par* function.

We further examined the location of the *par* locus within the 0.35-kb *Hae*III-*Hin*dIII fragment by subcloning the internal 0.26-kb *Hae*III-*Pst*I fragment. Using a similar approach, we cloned this DNA fragment into the repaired *Eco*RI site and the *Pst*I site of plasmid pDH5060. The phenotype of the resulting plasmid, pSYC730 (Fig. 6C), was again Par$^+$. This localized the *par* locus within the 0.26-kb *Hae*III-*Pst*I fragment.

Nucleotide sequence of the *par* fragments. The cloned *par* locus resides in the 0.26-kb *Hae*III-*Pst*I region in pOG2381. This fragment consists of the *par* sequence and a segment of unrelated sequence brought adjacent to *par* during the cloning process. We determined the nucleotide sequence of *par* cloned in pOG2381 and compared it with the *par* sequence in the 1.4-kb *Cla*I fragment in pLS11. The data are summarized in Fig. 7.

The two sequences share homology up to position 167, which is just beyond the *Bst*EII site. The sequence within the common fragment contains the *par* locus, as was shown by deletion mapping (see below). This region is relatively high in G+C content and has the following interesting

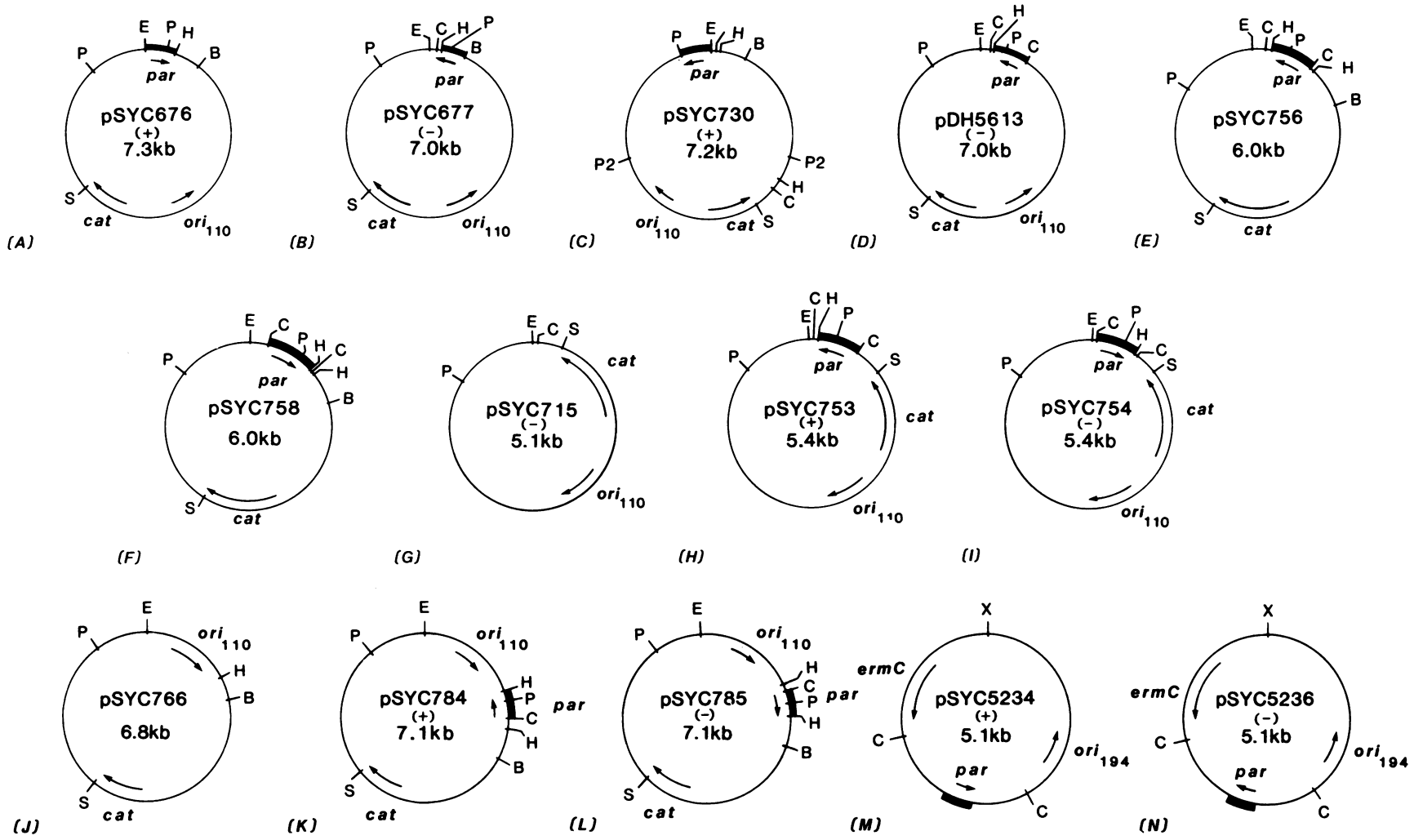

FIG. 6. Maps of plasmids used for the partition analysis. The orientation and location of the replication origin fragments from pUB110 (ori_{110}) and pBD15 (ori_{194}) and the cloned *par* sequence in these plasmids are shown. Restriction sites *Eco*RI (E), *Cla*I (C), *Hin*dIII (H), *Pst*I (P), *Pvu*II (P2), *Bam*HI (B), and *Stu*I (S) are drawn roughly in proportion. The estimated size and the observed plasmid partition phenotype [(+) for Par^+, (−) for Par^-] are shown in the center of each map. The direction of *ori* in pBD15 is reversed from that shown here on the basis of the determination of the sequence in the single-stranded plasmid for *B. subtilis* (see Discussion).

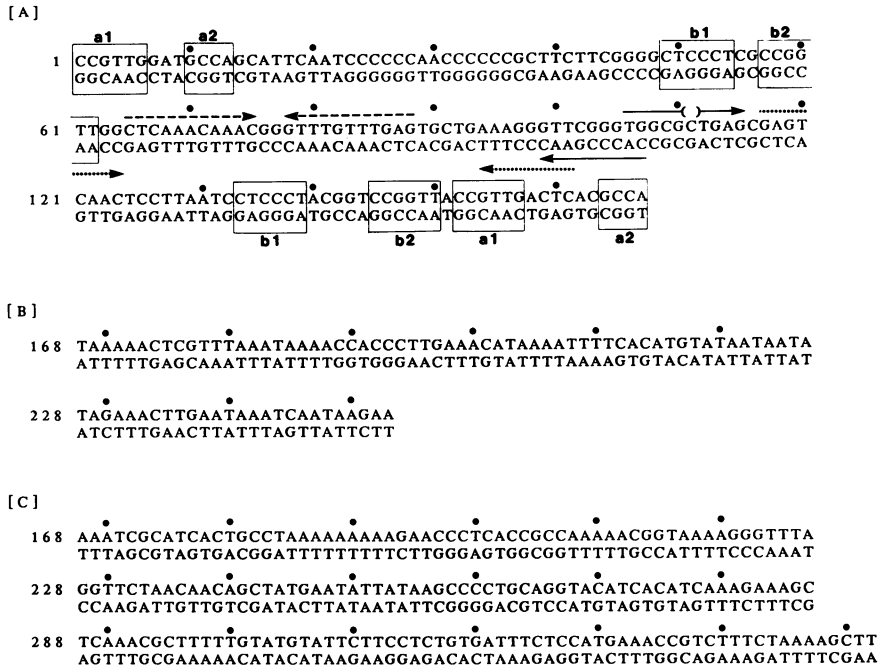

FIG. 7. Nucleotide sequence of the *par*-containing fragments. (A) Sequence of *par*; it is the sequence common to pLS11 and the cloned fragment in pOG2381. (B) Sequence found in pLS11 immediately downstream from *par*. (C) Sequence downstream from *par* in pOG2381. Nucleotides are numbered from the first position at the *Hae*III end. Repeated sequence (in boxes) and inverted sequences (pairs of arrows) are highlighted.

features: (i) there exist two perfect inverted repeats and one inverted repeat with a gapped nucleotide within one unit of the repeats; and (ii) there are two sets of direct repeats with internal mismatches. These features are highlighted in Fig. 7.

The *par* locus does not contain the replication function. *E. coli* plasmid pBR322 does not replicate in *B. subtilis*. Plasmid pJH101 is a pBR322 derivative carrying the *cat* gene derived from plasmid pC194 (8). It does not transform *B. subtilis* competent cells, because it lacks the necessary replication function. To test whether the *par* locus also carries the replication function, we cloned the 0.35-kb *par* fragment in plasmid pJH101 and transformed *B. subtilis* BD224 cells with the resulting plasmids.

We converted the *Bam*HI site in plasmid pSYC677 (Fig. 6B) to a *Cla*I site. The *par* fragment was excised from the resulting plasmid (pDH5613; Fig. 6D) by *Cla*I digestion and cloned into the unique *Cla*I site of pJH101. Plasmids were obtained that had the *par* sequence located on a *Cla*I-*Hin*dIII-*par*-*Cla*I fragment inserted in both orientations. These plasmids, pSYC756 and pSYC758 (Fig. 6E and F, respectively), were used to transform competent cells of BD224. No transformant was obtained with either plasmid, although thousands of Cm^r transformants were obtained in the control experiment with pOG2326 DNA. The failure of pSYC756 and pSYC758 plasmids to transform BD224 was most probably due to the lack of the necessary replication function(s) in these plasmids. We conclude from these data that the *par* locus does not contain the genetic elements required for plasmid replication in *B. subtilis*.

Expression of *par* is orientation dependent. The presence of the partition fragment on a plasmid does not always confer the Par^+ phenotype upon the plasmid. As described in the previous sections (Fig. 6), plasmids pOG2381 and pSYC1123 are Par^+; plasmids pOG2401 and pSYC1122, which contain the same inserts in the opposite orientation, are Par^-. In addition, plasmid pSYC676 is Par^+, but when the same 0.35-kb *Hae*III-*Hin*dIII fragment is cloned in a different region in pOG2326 (yielding plasmid pSYC677), the product exhibits the Par^- phenotype. To further investigate the effects of the neighboring sequences on the expression of the cloned *par* sequence, we made several constructions (described below).

Plasmid pDH5060 (Fig. 2) contains two *Cla*I sites. We deleted the small *Cla*I fragment in vitro that generated plasmid pSYC715 (Fig. 6G). The 0.35-kb *par* fragment from pDH5613 that is flanked by *Cla*I sites was excised with *Cla*I endonuclease. The purified *par* fragment was then inserted into the unique *Cla*I site in pSYC715 in both orientations; this generated plasmids pSYC753 and pSYC754 (Fig. 6H and I). The phenotype of BD224(pSYC753) was Par^+, and that of BD224(pSYC754) was Par^-.

Among the pairs of plasmids we examined, pSYC730, pSYC753, and pSYC754 were derived from pDH5060, whereas pOG2381, pOG2401, pSYC1122, pSYC1123, pSYC676, and pSYC677 were pOG2326 derivatives (Fig. 6). In both pOG2326 and pDH5060, the replication origin originates from plasmid pUB110, which is known to replicate unidirectionally (25). The direction of replication (shown in Fig. 2 and 6) is opposite to the direction of transcription of the *cat* gene derived from pC194 (14). We arbitrarily assigned the *Hae*III-*Pst*I orientation as the orientation of the *par* fragment (Fig. 4A and 6). The common feature that links the orientation of the *par* fragment to the Par phenotype among all the *par*-containing plasmids is the following. Whenever the orientation of *par* is the same as that of *cat*, the plasmids are phenotypically Par^+; when they are in the opposite orientation on the same plasmid, the plasmid is Par^-. The reverse is true for the orientation of the *par* fragment relative to that of the ori_{110} fragment in these plasmids. From the analysis of the structure of these plas-

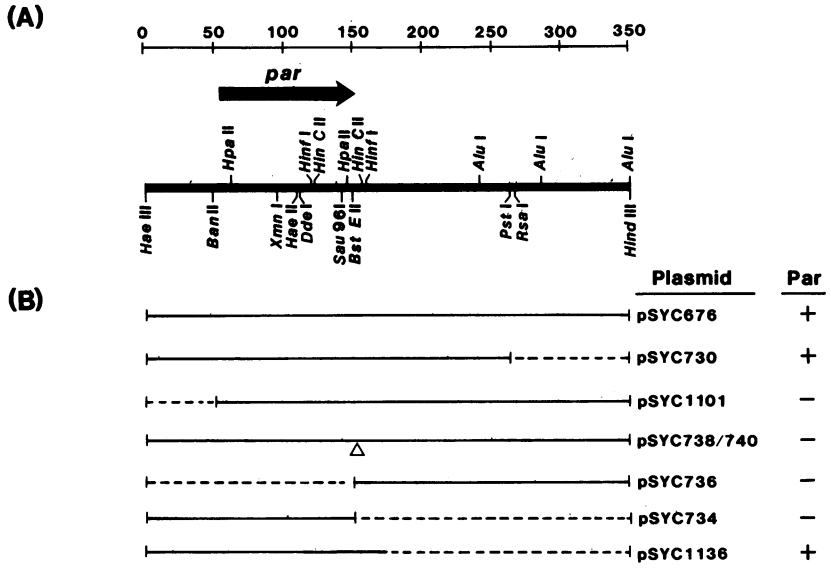

FIG. 8. Deletion mapping of *par*. (A) Restriction map of the 0.35-kb *Hae*III-*Hin*dIII fragment cloned in pOG2381. (B) Plasmids containing deletions (-----) or insertions (△) in the *par* fragment. The phenotypes of these plasmids are indicated.

mids, we conclude that the location of the *par* fragment in a plasmid is not critical, but that its orientation is important.

To further analyze the orientation dependence of *par*, we constructed new bifunctional vectors that allowed us to independently assess the effects of the orientation of the ori_{110} fragment and that of the *cat* fragment on *par* expression. Plasmid pJH101 carries the *cat* gene from pC194. We replaced the small *Eco*RI-*Cla*I region on pJH101 with the 1.4-kb *Eco*RI-*Hae*III fragment containing the replication origin from pUB110. This plasmid, designated pSYC766 (Fig. 6J), has the *ori* and *cat* genes in the same orientation and has a unique *Hin*dIII site. The *par* fragment in plasmid pSYC756 (Fig. 6E) was excised with *Hin*dIII and inserted into the *Hin*dIII site of pSYC766. The resulting plasmids, pSYC784 and pSYC785, carried the *par* fragment in a different orientation (Fig. 6K and L). pSYC784 was Par^+, and pSYC785 was Par^-. For all the plasmids studied (Fig. 6), the Par^+ phenotype was obtained only from plasmids which had the *par* oriented in the opposite direction to the pUB110-derived *ori*, independent of the orientation of the *cat* gene.

To determine whether the observed orientation dependence applies to other replicons, we constructed a different set of plasmids. Plasmid pE194 is an *S. aureus* plasmid that replicates in *B. subtilis*. It replicates unidirectionally (25). However, the precise direction of pE194 replication is controversial at present (see Discussion). We inserted the 1.4-kb *Cla*I fragment that contains the *par* from pLS11 into the *Cla*I site of pBD15, a copy number mutant of pE194 (12). The two resulting plasmids, pSYC5234 and pSYC5236, were Par^+ and Par^-, respectively (Fig. 6M and N). In the Par^+ plasmid pSYC5234, the *par* fragment was oriented in the same direction as that of the *ori* in pBD15 (see Discussion). We do not know whether partition of pBD15 is under genetic control or, if it is under genetic control, whether insertion at the *Cla*I site affects the partition of the pBD15 plasmid. However, we again observed the orientation-dependent expression in plasmids that carry the pLS11-derived *par* locus.

Localization of the cloned *par* sequence by mutation. The restriction map of the *par* fragment is shown in Fig. 8A. In an attempt to further define the sequence of the *par* locus, we made insertions and deletions in this fragment. Plasmid pSYC1101 (Fig. 8B) is derived from pOG2326. It carries the *par* fragment that includes the sequence from nucleotide position 55 to the downstream *Hin*dIII site. Plasmid pSYC1101 is similar to the Par^+ plasmid pSYC676, except that the region located between positions 1 and 54 in *par* is deleted. pSYC1101 was Par^-. Therefore we concluded that deletion of the first 54 base pairs (bp) in the *par* fragment removed some essential sequence from the *par* locus.

The *par* sequence in pSYC676 was digested with *Bst*EII (GGTNACC; occurs only once in the plasmid, at position 149), and the termini were repaired to generate flush ends. We subjected this DNA to the following different manipulations. (i) The linearized plasmid was digested with *Eco*RI and repaired by polymerase. The resulting large fragment was ligated to generate the deletion derivative pSYC736. (ii) Similarly, *Hin*dIII was used to generate the derivative pSYC734, deleting the sequence between *Bst*EII and *Hin*dIII. (iii) A 205-bp *Eco*RI fragment containing the *E. coli lac* operon promoter sequence was inserted, and pSYC740 was generated. (iv) The linearized fragment was ligated to generate pSYC738. This resulted in the loss of the *Bst*EII

TABLE 1. Plasmid copy number determination[a]

Plasmid	Size (kb)	Plasmid/chromosome ratio (%)	Estimated copy no.
pBD15	3.73	7.55	76
pDH5060	7.5	3.89	20
pSYC715	5.1	5.01	37
pSYC753	5.4	3.86	27
pSYC754	5.4	5.20	36

[a] Plasmid copy numbers (per chromosome) in strain BD224 were determined by the method of Gryczan et al. (12). Cultures were propagated in the presence of appropriate antibiotics. For the plasmid/chromosome ratio, we present the data as the average of two experiments. The size of the *B. subtilis* chromosome was taken as 3,750 kb.

site and, in theory, the insertion of five nucleotides at this location. The data are summarized in Fig. 8.

In addition, we also created a *Hin*dIII site at position 167 of the *par* sequence by using site-directed mutagenesis. The manipulation puts the A residue at position 167 as the first A residue in the *Hin*dIII site (AAGCTT). The 167-bp *par* sequence flanked by *Eco*RI and *Hin*dIII was then cloned into plasmid pOG2326 at the corresponding sites, resulting in plasmid pSYC1136.

The partition phenotypes for these plasmids are summarized in Fig. 8. Plasmids generated by manipulation that involved the *Bst*EII site were all Par$^-$. The smallest *par* segment that yielded a Par$^+$ plasmid was pSYC1136, which contained 167 bp of the *par* sequence. From these series of experiments we concluded that the *par* locus includes sequences to the left of position 54 and the sequence at the *Bst*EII site at position 148 to 154. Even a simple insertion of 5 bp at the *Bst*EII site (i.e., in pSYC738) inactivated the *par* locus.

Plasmid copy number is not increased by the *par* locus. The pLS11-derived *par* does not complement the missing replication function in pJH101, indicating the *par* is distinct from *ori*. It is possible that the *par* locus could confer the Par$^+$ phenotype by increasing the copy number of the replicating plasmids in *B. subtilis*. If plasmids partition by random distribution, this would significantly reduce the rate of plasmid loss during cell division. We therefore measured the plasmid copy numbers of some of the Par$^+$ and Par$^-$ plasmids (Table 1). The method we used (12) involves the determination of the mass ratio of plasmid to chromosomal DNA in a bacterial population and provides the deduced plasmid copy number. Plasmid pBD15, included here as a control, has a copy number of 76, which is close to that reported (12). Plasmid pSYC715 (37 copies per chromosome) has a higher copy number than that of its parent plasmid, pDH5060 (20 copies per chromosome). The reason for this is unclear at present; it could be related to the size difference between these plasmids. The *par*-containing pSYC715 derivatives, plasmids pSYC753 (Par$^+$) and pSYC754 (Par$^-$), have estimated copy numbers of 27 and 36, respectively. Therefore, the pLS11-derived *par* locus does not increase the plasmid copy number.

The pLS11-derived *par* functions only in *cis*. We initially identified the Par$^+$ plasmid pOG2381 by *cis* complementation. To test whether *par* functions in *trans*, we constructed the doubly transformed strain BD224(pSYC5191, pOG2326). Plasmid pOG2326 remained Par$^-$, and plasmid pSYC5191 was Par$^+$ in this doubly transformed strain. This result indicated that *par* does not complement in *trans*.

Since the expression of *par* appeared to be independent of its location on the plasmids, it suggests that transcription in the *par* locus from the neighboring region is probably not required for *par* to function. To confirm this, we cloned the constitutive *Bacillus penP* promoter adjacent to the *par* sequence on different plasmids. The results indicate that transcription from either end of the *par* sequence did not affect the Par phenotype (data not shown).

DISCUSSION

The pLS11-derived *par* is a *cis*-acting element. It does not complement in *trans*; therefore it probably does not specify a diffusible gene product. Expression of the *par* gene on a plasmid is determined by its orientation, not by its location, on the plasmid. The relative orientation of *par* to the replication origin fragment on the same plasmid determines the Par phenotype (Fig. 6). The orientation specificity is not due to the dependence on transcriptional activation. In the inactive orientation, *par* cannot be activated by transcription initiated from a promoter inserted adjacent to it.

The *par* from plasmid pLS11 does not contain the plasmid replication function. We have localized the *par* to a 167-bp sequence (Fig. 7A). Deletion analyses show that removal of the sequences of the first 54 bp (pSYC1101) or the last 15 bp (pSYC734) in this fragment inactivated the *par* function. Thus this locus spans beyond this 98-bp region. A computer search for homology between the 167-bp *par* and the plasmid sequences of pUB110 (19), pC194 (14), pE194 (13), and pT181 (15) was performed. We detected some short sequence homologies (less than 10 nucleotides); the significance, if any, of these short matches is unknown. However, no extended sequence homologous to *par* was found in these plasmid sequences.

The nucleotide sequence of the 167-bp par fragment contains several inverted repeats and direct repeats; the longer repeats are shown in Fig. 7A. These repeated sequences might be involved in interaction with other components that participate in plasmid partition. Although we do not understand the underlying mechanisms governing pLS11 partition, we can rule out several possibilities. We showed that a Par$^+$ plasmid does not have a higher copy number than its Par$^-$ counterpart. We also performed Southern blotting analysis with the 152-bp *Hae*III-*Bst*EII fragment as a probe. We found no homology between the *par* sequence and the chromosomal DNAs from *B. subtilis*, *B. amyloliquefaciens*, and *E. coli*. Thus it is unlikely that the Par$^+$ plasmid recombines with the chromosome during cell division to achieve partition proficiency. The orientation dependence of *par* expression suggests that it does not function as the site for resolving multimeric forms of the plasmid as does the *cer* locus in the ColE1 plasmid (27) or the *loxP* site in the P1 plasmid (5).

Several Par$^+$ plasmids have been constructed by molecular cloning of this *par* fragment in the Par$^-$ parental plasmids (such as pOG2326) that exhibit a high degree of instability. All of these resulting Par$^+$ plasmids showed extraordinary plasmid stability. On other hand, the pLS11-derived *par* does not confer partition proficiency in *E. coli*. This indicates that the *par* locus of pLS11 exhibits species specificity and is consistent with the notion that certain specific host components are involved in interacting with the *par* locus or with the *par* gene product.

Recently Te Riele et al. reported the detection of a single-stranded, strand-specific circular form of plasmid DNA in *Bacillus* and *Staphylococcus* spp. (29, 30). This single-stranded form represents about one-third of the plasmid copies for plasmids such as pE194 and pC221, whereas plasmids pUB110 and pBC16 generate very little single-stranded form (29). It was suggested that the single-stranded plasmid DNAs in *Bacillus* and *Staphylococcus* spp. are replication intermediates generated via a rolling-circle mode of DNA replication (10, 29, 30).

On the basis of the results of the experiments reported in this paper, we propose that the pLS11-derived *par* locus is a single-stranded site. This hypothesis explains the observation that *par* expression is transcription independent and orientation dependent.

How might a single-stranded site regulate plasmid partition? Since the single-stranded circular plasmid DNA appears to be a natural form of plasmid DNA in *Bacillus* spp., it is possible that *par* functions in this form of plasmid and

plays a key role in regulating plasmid partition. For example, one possibility is that a specific strand of the *par* sequence would be present in the single-stranded plasmid and would be available for interaction with other component(s). Alternative explanations are possible and cannot be ruled out at present.

Because pUB110 and its derivatives accumulate very little single-stranded form of plasmid DNA (29), we were unable to determine experimentally which strand of the *par* locus is present on the single-stranded plasmids derived from pUB110. The predominance of pUB110 in the double-stranded form may be due to efficient conversion of the single-stranded pUB110 plasmid to double-stranded DNA. Our proposal predicts that the pLS11-derived *par* will not exhibit the Par^+ phenotype on a replicon that is totally deficient in generating single-stranded DNA. The fact that pUB110 does not accumulate high levels of single-stranded plasmid DNA is not necessarily in conflict with our proposed model.

Noirot et al. (P. Noirot, M.-A. Petit, and S. D. Ehrlich, J. Mol. Biol., in press) determined the replication direction of pE194 in *Bacillus* spp. by identifying the strand that was displaced during replication (21). Their data indicate that the direction of pE194 replication is opposite to that reported previously (25) based on electron-microscopic analysis of replicating plasmid DNA molecules. Thus the Par^+ plasmid pSYC5234 (Fig. 6M) has the *par* and the pE194-derived *ori* fragments oriented opposite to each other, just as they are in all the pUB110-derived Par^+ plasmids. These data indicate that the par sequence that is present in the single-stranded form in the Par^+ plasmid pSYC5234 is the sequence of the lower strand shown in Fig. 7A. Thus our proposal predicts that this specific strand of the *par* fragment must be present in the single-stranded form of plasmid DNA for *par* to function.

Gruss et al. recently identified palindromic sequences (*palA*) in several staphylococcal plasmids. They found that *palA* mutant plasmids accumulated single-stranded plasmid DNA corresponding to the displaced plus strand in replication and proposed that the *palA* site is involved in the initiation of DNA synthesis of the lagging strand (10). The *Bacillus par* locus shares some common properties with the staphylococcal *palA* site: they are *cis*-acting sites, and the expression of these elements is dependent on their orientation. On the other hand, we found no significant sequence homology between *par* and the *palA* sites. Although the *palA* site is required for maintaining normal plasmid copy number and plasmid stability in *Staphylococcus* spp., it has no demonstrable function in *Bacillus* spp. (10). Therefore the *par* locus is functionally distinct from *palA*.

We also showed that pLS11 derivatives missing the *par* locus are still capable of replicating autonomously. However, these data do not rule out the possibility that *par* plays a nonessential role in plasmid replication, similar to that observed for the pSC101 *par* locus (31).

ACKNOWLEDGMENTS

We thank T. Tanaka for providing plasmid pLS11, D. Ho for construction of some of the plasmids used in these studies, and S. D. Ehrlich and R. P. Novick for providing information prior to publication. The excellent secretarial assistance of E. McCallan and S. Brown is acknowledged.

ADDENDUM IN PROOF

S. Bron et al. (S. Bron, P. Bosma, M. von Belkum, and E. Luxen, Plasmid, in press) recently reported that the genetic element required for maintaining plasmid pTA1060 stability is located on one or both of the small *Cla*I fragments of 1.45 and 0.20 kb. Since plasmid pTA1060 is the same as pLS11 (32), their deletion mapping result is consistent with our data reported here.

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