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Identification of Plasmid Partition Function in Coryneform Bacteria

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We have identified and characterized a partition function that is required for stable maintenance of plasmids in the coryneform bacteria *Brevibacterium flavum* MJ233 and *Corynebacterium glutamicum* ATCC 31831. This function is localized to a *Hin*dIII-*Nsp*V fragment (673 bp) adjacent to the replication region of the plasmid, named pBY503, from *Brevibacterium stationis* IFO 12144. The function was independent of copy number control and was not associated directly with plasmid replication functions. This fragment was able to stabilize the unstable plasmids in *cis* but not in *trans*.

Plasmids serve as powerful tools in the expression of a specific enzyme and/or metabolite in the cultivation of microorganisms when combined with recombinant DNA technology. However, in many industrial applications of this technology, the instability of a recombinant plasmid is a serious problem.

In *Escherichia coli*, several attempts have been made to overcome the problem mentioned above. Insertion of the partition locus of plasmid pSC101, *par*, could increase the stability of some plasmids (19), and the plasmids bearing the wild-type *valS* gene were well maintained in a *valS* mutant (18). We have also reported satisfactory stabilization of multicopy plasmids, such as pBR322 derivatives, by the inserted mini-F fragment of F factor (24–26).

Coryneform bacteria are gram-positive microorganisms, widespread in nature, that have been used for industrial fermentation of many metabolites, including L-glutamic acid (8) and L-lysine (13). We have been studying the coryneform strain *Brevibacterium flavum* MJ233. This strain has some useful characteristics, including the ability to metabolize ethanol as a sole carbon source, a rapid growth rate, and no autolysis under starvation conditions (21). We have also been studying *B. flavum* MJ233 for the production of chiral chemicals such as L-aspartic acid (21), L-isoleucine (28), and L-malic acid (27).

Recently, recombinant DNA techniques for these coryneform bacteria, including host-vector systems and transformation methods, have been developed (7, 12, 15, 23). We also reported the application of electroporation for the transformation of coryneform bacteria and demonstrated the possibilities for selection of transformants on minimal medium (MM) by auxotrophic complementation and high transformation frequency (9, 16).

However, there have been no studies on the stabilization of plasmids in coryneform bacteria; therefore, we attempted to construct an efficient stabilization system for recombinant plasmids in coryneform bacteria. In this study, we identified and characterized the DNA region for plasmid partition function derived from the plasmid in *Brevibacterium stationis* IFO 12144. This DNA region could increase the stability of plasmids in *B. flavum* MJ233 and *Corynebacterium glutamicum* ATCC 31831. This is the first report on the stabilization of plasmids in coryneform bacteria.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are described in Table 1. *B. flavum* MJ233 harbors plasmid pBY502 (about 45 kb in size); a cured strain was isolated. Plasmids pHSG398 and pHSG298, carrying the chloramphenicol or kanamycin resistance gene (Cm^r or Km^r) (20), were purchased from Takara Shuzo Co., Ltd., Kyoto, Japan.

Culture conditions. MM [glucose, 20 g; $(NH_4)_2SO_4$, 7 g; urea, 2 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 6 mg; $MnSO_4 \cdot 6H_2O$, 6 mg; biotin, 200 μg; thiamine-HCl, 200 μg; and deionized water, 1 liter (adjusted to pH 7.5 with NaOH)] was used for selection of transformants by auxotrophic complementation. AR medium (1 g of yeast extract and 1 g of Casamino Acids added to MM) was routinely used for growth. In selective media, 5 μg of chloramphenicol (Cm) per ml or 25 μg of kanamycin (Km) per ml was added. Cultivation was done with shaking (220 rpm, 70-cm stroke) at 33°C for *B. flavum* MJ233 or at 30°C for *C. glutamicum* ATCC 31831.

Preparation of plasmid DNA. Plasmid DNA was isolated by the alkaline lysis procedure (1).

Transformation by electroporation. The basic protocol was based on our previous methods (9, 16). A 0.5% inoculum from an overnight culture of *B. flavum* MJ233 in AR medium was inoculated into 100 ml of the same medium and grown with shaking. When the optical density at 610 nm of the culture reached 0.5, penicillin G (final concentration, 1 U/ml) was added. After incubation for 2 h at 33°C, the cells were harvested from 10 ml of culture broth by centrifugation at 4°C for 10 min at 6,000 rpm and were washed twice with 6 ml of chilled, deionized water and once with 6 ml of chilled transformation buffer (HG buffer), which contained 1 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and 10% glycerol (pH 7.4). The cells were suspended in the HG buffer to a final volume of 180 μl (approximately 5×10^{10} cells), mixed with plasmid DNA (1 μg), dissolved in 20 μl of HG buffer, and kept on ice for 2 or 3 min. HG buffer (0.8 ml) was added to the cuvette (0.2-cm electrode) containing the suspension, and the cuvette was placed in a Gene Pulser apparatus chamber (Bio-Rad Co., Ltd.). After one pulse (electric field strength, 12.5 kV/cm), the suspension was diluted by a fivefold volume of AR medium and incubated at 33°C for 1 h. Aliquots were spread on AR agar plates or MM agar plates containing 5 μg of chloramphenicol per ml or 25 μg of kanamycin per ml. Transformation for *C. glutamicum*

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Phenotype or description	Source or reference
Strains		
B. flavum MJ233C	A cured strain	9
B. flavum MJ233T	Tryptophan auxotroph isolated from MJ233C	9
C. glutamicum	ATCC 31831	American Type Culture Collection
Plasmids		
pBY503	Cryptic	16
pHSG398	pUC18 with Cm^r gene replacing Ap^r gene	20
pHSG298	pUC18 with Km^r gene replacing Ap^r gene	20
pCRY2	Cm^r	16; this study (Fig. 5)
pCRY2K	Km^r	This study (Fig. 5)
pCRY30	Cm^r	This study (Fig. 1)
pCRY30K	Km^r	This study (Fig. 5)
pCRY28	pCRY2 carrying the 673-bp fragment from pCRY38	This study (Fig. 5)
pCRY31	Cm^r	This study (Fig. 1)
pCRY32	Cm^r	This study (Fig. 3)
pCRY33	Cm^r	This study (Fig. 3)
pCRY34	Cm^r	This study (Fig. 3)
pCRY35	Cm^r	This study (Fig. 3)
pCRY36	Cm^r	This study (Fig. 3)
pCRY37	Cm^r	This study (Fig. 3)
pCRY38	Cm^r	This study (Fig. 3)
pCRY39	Cm^r	This study (Fig. 3)
Mini-F-*trp*	Mini-F carrying *E. coli trp* operon	24
pCRY30*trp*	pCRY30 carrying *E. coli trp* operon	This study (Fig. 5)
pCRY38*trp*	pCRY30*trp* carrying the 673-bp fragment from pCRY38	This study (Fig. 5)

ATCC 31831 was done by the same method, except that the culture was kept at 30°C.

Plasmid stability test. Cells harboring plasmids were grown to the stationary phase in AR medium containing antibiotics, were inoculated into AR medium at approximately 50 cells per ml, and were grown for the average generations indicated, and then aliquots of the cultures were diluted and spread on AR plates. After overnight incubation at 33°C, the phenotypes of 100 colonies from each plate were examined by transferring the colonies with toothpicks onto selective plates containing antibiotics. Cell concentrations were determined before and after the cultivation. Experiments repeated at least three times gave essentially the same results. The stability test for *C. glutamicum* ATCC 31831 was done by the same method, except that the culture was kept at 30°C.

Plasmid copy number determination. The method for determining plasmid copy number was that of Gryczan et al. (5).

Chloramphenicol acetyltransferase assay. Chloramphenicol acetyltransferase activity was determined colorimetrically (17) and was expressed as nanomoles of chloramphenicol acetylated per minute at 37°C per milligram of cellular protein. Protein determination was done by the method of Bradford with the Bio-Rad assay kit.

DNA sequencing. Specific restriction fragments were cloned into pUC118 or pUC119 (22) and sequenced by the dideoxy chain termination method of Sanger et al. (14). DNA was labeled with [α-^{32}P]dATP (400 Ci/mmol). Analysis of sequence data was performed by using the GENETYX programs (Software Development Co., Ltd., Tokyo, Japan).

Nucleotide sequence accession number. The nucleotide

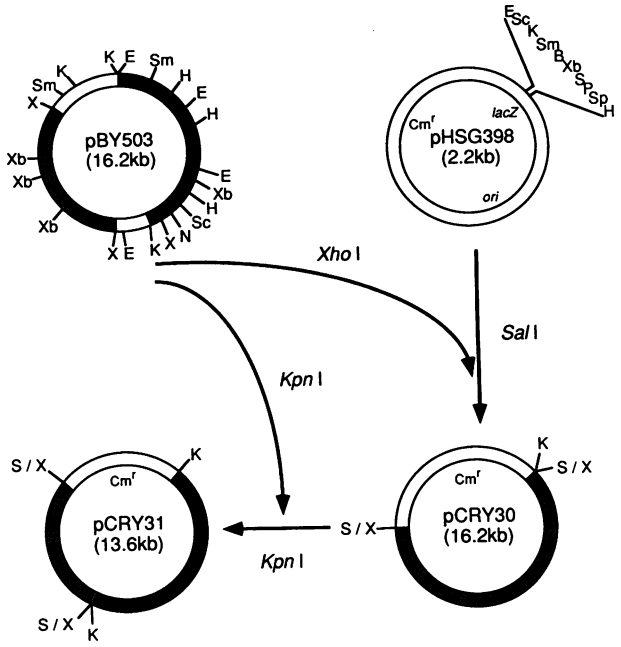

FIG. 1. Physical maps of plasmid pBY503 and its derivatives. Plasmid pCRY30 was constructed by inserting a 4.0-kb *Xho*I fragment carrying the replication function of plasmid pBY503 into pHSG398 at the *Sal*I site. Plasmid pCRY31 was constructed by inserting a 7.4-kb *Kpn*I fragment carrying the partition function of plasmid pBY503 into pCRY30 at the *Kpn*I site. Symbols: ■, partition function of pBY503; ▩, the replication function of pBY503. Restriction site abbreviations are as follows: E, *Eco*RI; Sc, *Sac*I; K, *Kpn*I; Sm, *Sma*I; B, *Bam*HI; Xb, *Xba*I; S, *Sal*I; P, *Pst*I; Sp, *Sph*I; H, *Hin*dIII; N, *Nsp*V; and A, *Acc*I.

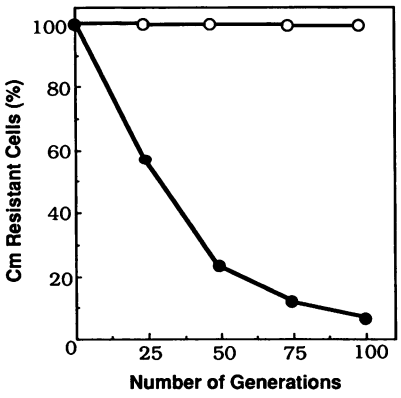

FIG. 2. Stability of plasmids pCRY30 and pCRY31 under nonselective conditions. The plasmid stability test is described in Materials and Methods. The cells for the stability test were obtained from 25, 50, 75, and 100 generations of growth. Symbols: ○, *B. flavum* MJ233C harboring pCRY31; ●, *B. flavum* MJ233C harboring pCRY30.

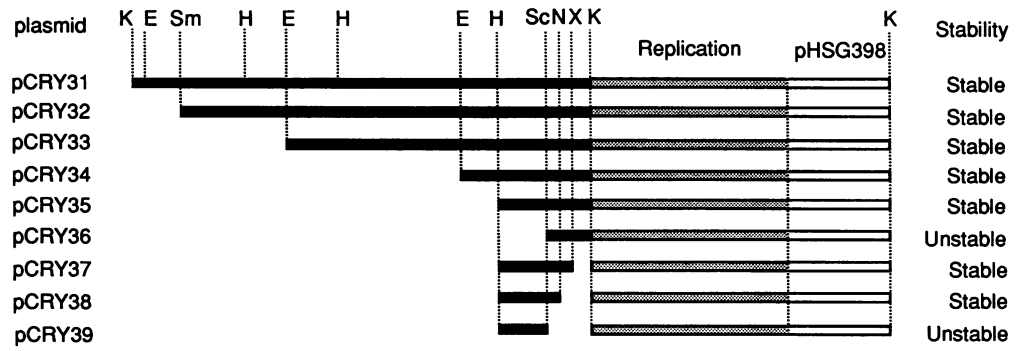

FIG. 3. Stability of deletion plasmids derived from plasmid pCRY31. The plasmid stability test is described in Materials and Methods. Symbols and restriction site abbreviations are as described in the legend to Fig. 1. Plasmid pCRY32 was obtained from plasmid pCRY31 with *Kpn*I and *Sma*I. Plasmids pCRY33 and pCRY34 were obtained from plasmid pCRY31 with *Kpn*I and *Eco*RI. Plasmids pCRY35 and pCRY36 were obtained from plasmid pCRY31 with *Kpn*I and *Hin*dIII and with *Kpn*I and *Sac*I, respectively. Plasmids pCRY37, pCRY38, and pCRY39 were obtained from plasmid pCRY35 with *Kpn*I and *Xho*I, with *Kpn*I and *Nsp*V, and with *Kpn*I and *Sac*I, respectively.

sequence in this paper has been assigned GenBank/EMBL accession no. D00661.

RESULTS

Identification of plasmid partition function of the pBY503 plasmid. We have constructed plasmid pCRY30, which is capable of replication in *B. flavum* MJ233C, consisting of the *Xho*I 4.0-kb fragment derived from plasmid pBY503 and the 2.2-kb fragment of plasmid pHSG398 containing the chloramphenicol resistance gene (Fig. 1). However, this plasmid could not be maintained stably under nonselective culture conditions. Plasmid pCRY31, which was constructed by insertion of the 7.4-kb *Kpn*I fragment from plasmid pBY503 into plasmid pCRY30, showed high plasmid partitioning under nonselective culture conditions (Fig. 2). Therefore, to determine the minimal region necessary for plasmid partition of the 7.4-kb *Kpn*I fragment, we constructed various deletion plasmids from plasmid pCRY31 and examined the stability of these plasmids in *B. flavum* MJ233C (Fig. 3). The plasmids pCRY36 and pCRY39 were unstable, and the rates of segregation were similar to that of plasmid pCRY30 (Fig. 2). The other plasmids were as stably maintained as pCRY31, and all the stable plasmids examined so far carried the 0.67-kb *Hin*dIII-*Nsp*V fragment within the 7.4-kb *Kpn*I fragment (Fig. 3); we concluded that the function for plasmid partition was located within this 0.67-kb fragment of plasmid pBY503.

Properties of the partition locus. Since even plasmids lacking the plasmid partition mechanism might be maintained relatively stably in high-copy-number plasmids, we measured the difference of copy number between plasmid pCRY30 and plasmid pCRY38 as the amount of covalently closed circular plasmid DNA per chromosomal equivalent. The copy number of the stable plasmid pCRY38 was similar to that of the unstable plasmid pCRY30 (Table 2). The levels of chloramphenicol acetyltransferase in growing cells were also equivalent. These results suggested that the stabilization of plasmid pCRY38 by the 0.67-kb fragment was not due to an increase in plasmid copy number. We concluded that the stabilization of plasmid seemed to be due to a plasmid partition mechanism encoded by this fragment.

TABLE 2. Determination of plasmid copy number

Plasmid	Copy number[a]	Chloramphenicol acetyltransferase activity[b]
pCRY30	4.6	10.2
pCRY38	4.7	11.2

[a] The average copy number of covalently closed circular plasmid DNA per chromosomal equivalent was calculated from the sizes of the plasmid (pCRY30, 6.3 kb, or pCRY38, 6.9 kb) and the chromosome (4.2×10^3 kb).

[b] Expressed as nanomoles of chloramphenicol acetylated per minute at 37°C per milligram of protein.

The nucleotide sequence of the 0.67-kb (673-bp) fragment is shown in Fig. 4. The GENETYX computer programs were used to analyze the partition region sequence for possible open translational reading frames and for secondary structure. This analysis indicated that no complete protein was encoded by this fragment. Furthermore, no region of possible secondary structure was detected.

The previously constructed plasmid pCRY2 (about two copies per chromosome) (16), consisting of the 4.1-kb *Hin*dIII fragment of plasmid pBY502 from *B. flavum* MJ233 and a 2.2-kb fragment of pHSG398 (Fig. 5), was found to be compatible with plasmid pCRY30 but was more unstable than plasmid pCRY30 in *B. flavum* MJ233C (data not shown). To test whether the partition function of pBY503 is able to stabilize this plasmid, we constructed plasmid pCRY28 by introducing the 673-bp fragment from plasmid pCRY38 into plasmid pCRY2 (Fig. 5) and examined the stability of this plasmid. Plasmid pCRY28 was as stably

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AAGCTTGCACATCAAAACGGCAATGTCAGCCCCATTGAGGTTCACGAGTTTCCGATTGATGCAATGCTACAAGCTCATGAGATAGCTGAGCTTGGCACGA
                                                                                                    100
CTTCGGCGCCAATATTCAACAGCTTGAGGTATCTCTTGGCGATGATGCAGTCGTCATCGTCGACTGTGTAGTACCCTTCGATGGCTCGTCGATGTCTGTTG
                                                                                                    200
AACCAACACGTCGAACCCTTTTGGCTATCAATGCGCCGACCATCATGTTTGGCTGTGGCCGAGAACGGATGAACCTATGTATCAAGAAATGTTGAACAAG
                                                                                                    300
GTAGGCGTTAAAGCGACCCGCAAGAGCGCTGCCATAGGTTATAGCGAGGATGCTCAGTGCTGCCGTATTCCCGATGATATGCCAGATTATGAAGCGTTGG
                                                                                                    400
CCTCTGAGCTAATTCAGTAAACGATGCTCGCGGCGGGTGAAATAACGCCAATGAACGGGGAACTGTATAGCTGTAAATACAGTACCGTAAATACAGCAG
                                                                                                    500
CTAGTTCTTATGCTTTTTTAGCTCTGCTTCTACGGCGTCGTGAATAATCTCTCGCATGGTCACAGACTGCAAAGCTGCGGCACTTGTGAGCTCAACGGTG
                                                                                                    600
AAGACGTGAATCAAGCTCCACGGTGAGCTTGACAGTATGATCGCTTTGATTGCGTGAGGCAAAGGCTTTCGAA
                                                                                                    670
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FIG. 4. Nucleotide sequence of the partition region from pBY503. The nucleotide sequence of the *Hin*dIII-*Nsp*V fragment derived from pBY503 is shown. The *Hin*dIII cleavage site has been assigned the 5′ end (+1) in this figure, and the sequence is numbered toward the *Nsp*V cleavage site.

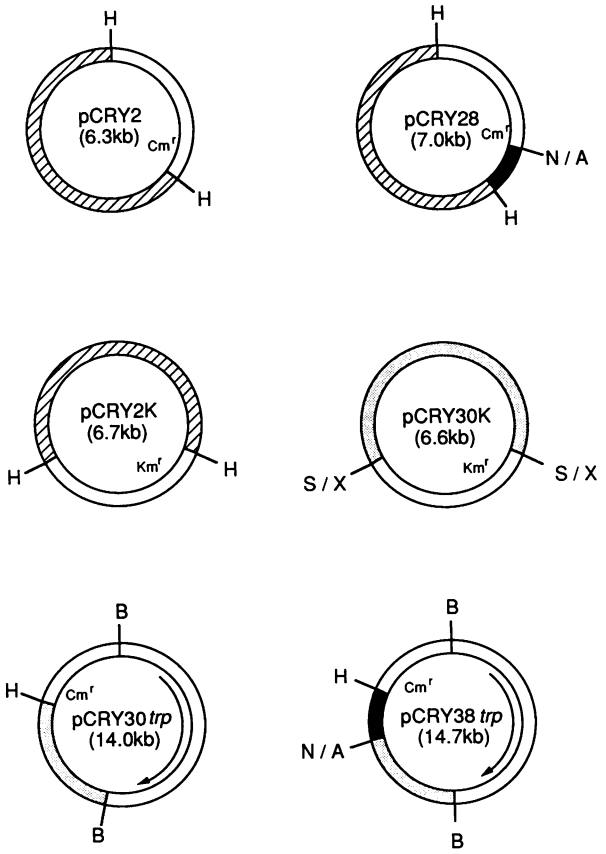

FIG. 5. Physical maps of plasmids used for the stability test. Plasmid pCRY2 was described in the text, and plasmid pCRY28 was constructed by inserting a 673-bp *Hin*dIII-*Nsp*V fragment carrying the partition function of plasmid pCRY38 into pCRY2 at the *Hin*dIII and *Acc*I sites. Plasmids pCRY2K and pCRY30K were constructed by inserting a 4.1-kb *Hin*dIII fragment of pCRY2 or a 4.0-kb *Xho*I fragment of pCRY30 into pHSG298 at the *Hin*dIII or the *Sal*I site. Plasmid pCRY30*trp* was constructed by inserting a 7.4-kb *Sal*I-*Xho*I fragment carrying the tryptophan operon of plasmid mini-F-*trp* into pCRY30 at the *Bam*HI sites with the aid of a *Bam*HI linker. Plasmid pCRY38*trp* was constructed by inserting a 673-bp *Hin*dIII-*Nsp*V fragment carrying the partition function of plasmid pCRY38 into pCRY30*trp* at the *Hin*dIII and *Acc*I sites. Arrows indicate the direction of transcription of the *trp* operon. Symbols: ▨, replication function of pBY502; ■ and ▥ and restriction site abbreviations, as described in the legend to Fig. 1.

maintained as plasmid pCRY38 and was stably compatible with plasmid pCRY38 (data not shown).

To determine whether the 673-bp fragment could reverse instability of the plasmid in *trans*, we constructed plasmids pCRY2K and pCRY30K containing the kanamycin resistance gene (Fig. 5). We introduced plasmid pCRY2K into cells of *B. flavum* MJ233C containing pCRY38 and examined the stability of plasmid pCRY2K for the kanamycin resistance phenotype. No detectable effects by the resident plasmid pCRY38 on the stability of plasmid pCRY2K could be observed, and similar effects on the stability of plasmid pCRY30K were obtained when plasmid pCRY30K was introduced into cells containing plasmid pCRY28 (Table 3). These results indicated that a *cis*-acting effect was likely.

To test whether the partition locus of plasmid pBY503 could function in other bacteria, we introduced plasmid

TABLE 3. *trans* complementation analysis by the partition locus in *B. flavum* MJ233C

Plasmid tested for stability	Resident plasmid	% Plasmid-containing cells[a]
pCRY2K	None	5
pCRY2K	pCRY38	4
pCRY30K	None	42
pCRY30K	pCRY28	43

[a] The plasmid stability test was done as described in Materials and Methods, except that cultivation was done in the presence of chloramphenicol. The cells for the stability test were obtained from 25 generations of growth.

pCRY30 or pCRY38 into *C. glutamicum* ATCC 31831 as the host strain and examined the stability of the plasmid under nonselective culture conditions. Plasmid pCRY30 was unstable, and the rates of segregation were similar in *B. flavum* MJ233C (Fig. 3), but plasmid pCRY38 was stably maintained in this strain (Table 4). Also, the copy number levels of between pCRY30 and pCRY38 in both *C. glutamicum* ATCC 31831 and *B. flavum* MJ233C were similar. Plasmid pCRY38 was also tested for its plasmid-stabilizing phenotype in *E. coli* HB101. The segregation rates were similar to that of plasmid pCRY30, and both were found to be unstable (data not shown). Hence, we concluded that the partition locus of plasmid pBY503 does not function in *E. coli*.

In the case of *trp* operon-bearing plasmids, high expression of the *trp* operon reduced plasmid stability in *E. coli* (6). When we constructed plasmid pCRY30*trp* consisting of the pCRY30 plasmid and the tryptophan operon from *E. coli* K-12 chromosome (Fig. 5), we found that this plasmid was more unstable than plasmid pCRY30 in *B. flavum* MJ233T under nonselective culture conditions (Table 5). To test whether the partition locus of plasmid pBY503 could stabilize pCRY30*trp*, we introduced the 673-bp fragment into plasmid pCRY30*trp* and examined the stability of this plasmid in *B. flavum* MJ233T. The resulting plasmid, pCRY38*trp* (Fig. 5), could be stably maintained, and simultaneously, loss of Trp^+ and Cm^r might be effectively suppressed (Table 5).

DISCUSSION

In this study, we identified and characterized a plasmid partition function in coryneform bacteria. This function, derived from plasmid pBY503 from *B. stationis* IFO 12144, showed the stable partitioning of plasmids to daughter cells during cell division and depends on a specific plasmid DNA locus. Using the 673-bp *Hin*dIII-*Nsp*V fragments of plasmid

TABLE 4. Stability of pCRY30 and pCRY38 in *C. glutamicum* ATCC 31831

Plasmid	Copy number[a]	% Plasmid-containing cells[b] after generation:		
		0	25	50
pCRY30	4.5	100	46	20
pCRY38	4.6	100	>95	>95

[a] Calculation of average copy number of covalently closed circular plasmid per chromosomal equivalent was done as described in footnote *a* of Table 2.
[b] The plasmid stability test was described in Materials and Methods.

TABLE 5. Stability of pCRY30*trp* and pCRY38*trp* in *B. flavum* MJ233T

Plasmid	Copy number[a]	Phenotype distribution (%)[b]			
		Trp^+ Cm^r	Trp^+ Cm^s	Trp^- Cm^r	Trp^- Cm^s
pCRY30*trp*	4.6	5	0	0	95
pCRY38*trp*	4.5	98	0	0	2

[a] The average copy number of each covalently closed circular plasmid per chromosomal equivalent was calculated from the sizes of the plasmid (pCRY30*trp*, 14.0 kb, or pCRY38*trp*, 14.7 kb) and the chromosome (4.2×10^3).

[b] Cells of *B. flavum* MJ233T carrying the plasmids indicated, which were grown in MM to the stationary phase, were inoculated at approximately 50 cells per ml, grown at 33°C for 50 generations, and then plated on AR plates with appropriate serial dilutions. After overnight incubation at 33°C, 100 colonies from each plate were examined for their phenotypes by transferring them with toothpicks to selective plates (MM, MM plus chloramphenicol, and MM plus tryptophan). Trp^+, tryptophan prototrophy; Trp^-, tryptophan auxotrophy; Cm^r, chloramphenicol resistance; Cm^s, chloramphenicol sensitivity.

pBY503, we have also shown that the function is physically and functionally separate from the replication and copy number control functions of the plasmid.

The 673-bp fragment restored partition function in *cis* but not in *trans*. Several reports have shown that the partition function, termed *par*, of pSC101 (10) in *E. coli* or pLS11 (2, 3), also referred to as pPOD2000 (4) in *Bacillus subtilis*, restored partition ability only in *cis*.

Partitioning of pSC101 was not dependent on the location or orientation on the plasmid of the reintroduced *par* fragment, but that of pLS11 was orientation dependent. Our preliminary data suggested that the partition function of plasmid pBY503 was also orientation dependent (data not shown).

No encoded protein was found in either the 167-bp fragment from pLS11 (3) or the 375-bp fragment from pSC101 (11); the partition locus of the 673-bp fragment from plasmid pBY503 also did not encode a complete protein. There was no significant homology between the 673-bp fragment of plasmid pBY503 and the 375-bp fragment of pSC101 or the 167-bp fragment of pLS11 at the nucleotide sequence level.

Meacock and Cohen proposed that distribution of plasmid molecules (in the case of pSC101) between daughter cells at cell division was mediated by interaction of this DNA locus with other cellular components of the partitioning system, such as the cytoplasmic membrane, and might be initiated by duplication of the *par* locus (10). We are also interested in plasmid partition, especially interaction of the DNA locus with cytoplasmic membrane, and studies are now in progress.

In this study, we constructed an efficient stabilization system for recombinant plasmids in the coryneform bacteria *B. flavum* MJ233 and *C. glutamicum* ATCC 31831 and demonstrated the system's use for stabilization of a plasmid bearing the tryptophan operon. We believe that this will facilitate the molecular design of high-expression vectors and highly stable cloning vectors for expression of foreign genes in coryneform bacteria for industrial purposes.

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