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 HindIII-NspV 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.

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' or Km') (20), were purchased from Takara Shuzo Co., Ltd., Kyoto, Japan.

Culture conditions. MM [glucose, 20 g; (NH₄)₂SO₄, 7 g; urea, 2 g; KH₂PO₄, 0.5 g; K₂HPO₄, 0.5 g; MgSO₄·7H₂O, 0.5 g; FeSO₄·7H₂O, 6 mg; MnSO₄·6H₂O, 6 mg; biotin, 0.5 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 × 10¹⁰ 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

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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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 pHCI18 or pUC119 (22) and sequenced by the dideoxy chain termination method of Sanger et al. (14). DNA was labeled with [α-32P]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
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 XhoI 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 KpnI 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 KpnI 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 HindIII-NspV fragment within the 7.4-kb KpnI 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.

The previously constructed plasmid pCRY2 (about two copies per chromosome) (16), consisting of the 4.1-kb HindIII 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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Copy number</th>
<th>Chloramphenicol acetyltransferase activity</th>
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<tbody>
<tr>
<td>pCRY30</td>
<td>4.6</td>
<td>10.2</td>
</tr>
<tr>
<td>pCRY38</td>
<td>4.7</td>
<td>11.2</td>
</tr>
</tbody>
</table>

* 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 × 10^6 kb).

* Expressed as nanomoles of chloramphenicol acetylated per minute at 37°C per milligram of protein.

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 KpnI and SmaI. Plasmids pCRY33 and pCRY34 were obtained from plasmid pHSG398 with KpnI and EcoRI. Plasmids pCRY35 and pCRY36 were obtained from plasmid pCRY31 with KpnI and HindIII and with KpnI and NspV, respectively. Plasmids pCRY37, pCRY38, and pCRY39 were obtained from plasmid pCRY35 with KpnI and XhoI, with KpnI and NspV, and with KpnI and SacI, respectively.

FIG. 4. Nucleotide sequence of the partition region from pBY503. The nucleotide sequence of the HindIII-NspV fragment derived from pBY503 is shown. The HindIII cleavage site has been assigned the 5′ end (+1) in this figure, and the sequence is numbered toward the NspV cleavage site.
Plasmid pCRY2 was described in the text, and plasmid pCRY28 was constructed by inserting a 673-bp HindIII-NspV fragment carrying the partition function of plasmid pCRY38 into pCRY2 at the HindIII and AccI sites. Plasmids pCRY2K and pCRY30K were constructed by inserting a 4.1-kb HindIII fragment of pCRY2 or a 4.0-kb XhoI fragment of pCRY30 into pHSG298 at the HindIII or the SalI site. Plasmid pCRY30trp was constructed by inserting a 7.4-kb SalI-XhoI fragment carrying the tryptophan operon of plasmid mini-F-trp into pCRY30 at the BamHI sites with the aid of a BamHI linker. Plasmid pCRY38trp was constructed by inserting a 673-bp HindIII-NspV fragment carrying the partition function of plasmid pCRY38 into pCRY30trp at the HindIII and AccI 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.

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 HindIII-NspV fragment carrying the partition function of plasmid pCRY38 into pCRY2 at the HindIII and AccI sites. Plasmids pCRY2K and pCRY30K were constructed by inserting a 4.1-kb HindIII fragment of pCRY2 or a 4.0-kb XhoI fragment of pCRY30 into pHSG298 at the HindIII or the SalI site. Plasmid pCRY30trp was constructed by inserting a 7.4-kb SalI-XhoI fragment carrying the tryptophan operon of plasmid mini-F-trp into pCRY30 at the BamHI sites with the aid of a BamHI linker. Plasmid pCRY38trp was constructed by inserting a 673-bp HindIII-NspV fragment carrying the partition function of plasmid pCRY38 into pCRY30trp at the HindIII and AccI 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.

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

<table>
<thead>
<tr>
<th>Plasmid tested for stability</th>
<th>Resident plasmid</th>
<th>% Plasmid-containing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCRY2K</td>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td>pCRY2K</td>
<td>pCRY38</td>
<td>4</td>
</tr>
<tr>
<td>pCRY30K</td>
<td>None</td>
<td>42</td>
</tr>
<tr>
<td>pCRY30K</td>
<td>pCRY28</td>
<td>43</td>
</tr>
</tbody>
</table>

* 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 pCRY30trp 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 pCRY30trp, we introduced the 673-bp fragment into plasmid pCRY30trp and examined the stability of this plasmid in B. flavum MJ233T. The resulting plasmid, pCRY38trp (Fig. 5), could be stably maintained, and simultaneously, loss of Trp+ and CmR 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 HindIII-NspV fragments of plasmid

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Copy numbera</th>
<th>% Plasmid-containing cellsb after generation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>pCRY30</td>
<td>4.5</td>
<td>100</td>
</tr>
<tr>
<td>pCRY38</td>
<td>4.6</td>
<td>100</td>
</tr>
</tbody>
</table>

* Calculation of average copy number of covalently closed circular plasmid per chromosomal equivalent was done as described in footnote a of Table 2.

* The plasmid stability test was described in Materials and Methods.
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 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 Bacillus flavidum 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.

REFERENCES


