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High-Expression of a Target Gene and High-Stability of the Plasmid

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INTRODUCTION

In the chemical industry, enzymes are commonly used a biocatalysts for several reasons.

1. They are chiral catalysts.
2. They have high substrate specificity, and
3. They are effective under mild conditions.

However, in view of the fact that most enzymes are too expensive to use in large-scale commercial processes, the widespread use of enzymes has yet to be achieved.

Expression vector systems can be of help to solve this problem. The expression of a target gene in a bacterial cell is a recent development that has revolutionized molecular biology. It may well have a major impact on the chemical industry, since this technology makes it possible to produce enzymes at a far more reasonable cost. The important events that have triggered the development of techniques in cloning and expressing of target genes in bacteria include the following:

1. The discovery of plasmids that can replicate in many copies within bacterial cells,

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2. The development of transformation protocols that enable introduction of plasmids into cells with high efficiency,
3. The discovery of restriction endonucleases that allows the cutting of DNA at specific sequences, and
4. The discovery of DNA ligases.

These techniques make it possible to express a target gene and produce its protein in bacterial cells, as long as the following requirements are fulfilled. Firstly, the gene must be placed downstream from a promoter sequence to permit transcription. Secondly, ribosomes must be able to bind to the RNA transcript and initiate translation at the start of the cloned gene. Other factors can also affect efficiency of this expression, including the choice of promoter, sequence of the ribosome-binding site, plasmid copy number, presence of transcription terminators, choice of bacterial host, and the conditions under which the recombinants are grown. By paying attention to these factors, an expression vector system with high-level production of a target enzyme can be achieved.

Having achieved high-level production in laboratory scale culture, other factors need to be considered when scaling-up for industrial processes. Following optimization of target gene expression, it is necessary to consider the effects of the gene product on the bacterium harboring recombinant plasmid. The increased level of target gene expression results in reduction of cell growth rates. Also by random distribution of plasmids to daughter cells, plasmid-free cells are produced. As a consequence, a cell culture loses productivity. This phenomenon of recombinant plasmid instability is a serious problem for large-scale cultivation. To prevent such a problem, it is desirable to minimize the target gene expression until the bacteria are introduced into the final fermentation vessel. Several attempts have been made to overcome this problem. One strategy is to use antibiotics. Another is to use some mutant hosts which are nonviable without certain proteins on the recombinant plasmid. However, antibiotics add considerably to the cost of the medium and the stabilizing effects found in the mutant host is difficult to evaluate in terms of practical applications.

To solve this problem more practically, a plasmid partitioning mechanism can be employed. An inserted plasmid partitioning locus can stabilize an otherwise unstable recombinant plasmid that is highly expressing a target gene. This region effectively suppresses loss of the whole plasmid, that is easy to understand when one considers the function of active plasmid partitioning locus. The application of active partitioning locus has enabled both high-expression of a target gene and high-stability of plasmid for large-scale cultivation in chemical industry.

In this article, we will:

1. briefly outline an efficient target gene expression system and
2. describe plasmid partitioning function and its use for large-scale cultivation.

High-Expression Foreign Gene System

To express a target gene at a high-level, transcription and translation should be maximized. There are four ways to maximize transcription and translation:

1. utilization of gene dosage effects,
2. amplification of transcripts,
3. optimization of translation, and
4. reduction of protein degradation.

(1) Utilization of Gene Dosage Effect

The Plasmid Copy Number

An important factor for high-level expression is the copy number of the expression vector. High copy number plasmids are commonly used. Plasmid pBR322 and its derivatives are typical examples of high copy number plasmids, producing >20 copies per cell (*1*). To increase the copy number, many plasmids with a runaway-type replicon (*2–5*), whose copy number can be increased by some conditions such as temperature shifting, were developed, and for many peptides and proteins, production was enhanced using such plasmids (*6,7*).

Tandem Repeat of a Gene

This is another method for high-level expression of the target gene without increasing the copy number of expression plasmid. Using a high-level expression vector, the target gene with a ribosome binding sequence is inserted tandemly into the expression vector to enhance the productivity.

Shibui et al. (*8*) constructed the plasmid pUS(CAT)n (n = 1, 2, 3, ·····) for the expression of chloramphenicol acetyltransferase (CAT) under the control of the *tac* promoter to demonstrate the validity of the above method. *E. coli* harboring a CAT expression plasmid, $pUS(CAT)_1$, that had one CAT gene inserted, was able to produce CAT at the level of only 4% of the total cellular protein. However, *E. coli* harboring a CAT expression plasmid, $pUS(CAT)_4$, that had four directly repeated CAT genes inserted, could synthesize CAT up to 16% of the total cellular protein.

Watanabe et al. (*9*) reported glutathione production in *E. coli* with tandemly polymerized genes. Also, Deutch et al. (*10*) inserted a 2.9 kb *E. coli pro*BA region, that encodes the first two enzymes of the proline biosynthetic pathway, directly repeated into an expression vector. In both cases, successful high-level expression of the target genes was observed.

High-level production of peptides or proteins, however, is basically disadvantageous to the host bacterium. The copy number of the plasmid, that express the target gene, is generally lower than that of the original vector. In some cases, a recombinant plasmid tandemly inserted target gene undergoes homologous recombination between the inserted genes.

For these reasons, even using such runaway-type replicons, plasmid cannot be increased or may be unstable in many cases.

(2) Amplication of Transcript

Use of Strong Promoters

An important element that is necessary for high-level expression of a target gene is the promoter. Sequences of a large number of promoters in *E. coli* are known. Promoters are divided into two types in terms of expression mechanism; constitutive expression and controllable expression. Constitutive promoters include 1pp (*11*), *bla* (*12*), and phage T5 P25 promoters (*13,14*), which are relatively strong. Controllable promoters include *lac* (*15,16*), *lacUV5* (*17*), *trp* (*18*), *tac* (*19,20*), and lambda P_L (*21*) promoters, that can be induced under certain conditions. Comparison of the known sequences of promoters (*22*) has revealed two consensus regions; 5'TTGACA3' at the -35 region (*23*) and 5'TAT-AAT3' at the -10 region (*24,25*).

Strong and controllable promoters are useful in genetic engineering. A good example of a strong and controllable promoter is *tac*, that was constructed as a fusion promoter of the *trp* (the -35 region) and the *lacUV5* (the -10 region) (*19,20*). The -35 region of the *trp* promoter and the -10 region of the *lacUV5* promoter have the same sequence of *E. coli* promoter consensus. The *tac* promoter can be under the control of the *lacI* gene (*26*) and is induced by the addition of IPTG. On the basis of these factors, *tac* is commonly used as a strong and controllable promoter for the expression of many target genes.

Further studies have been carried out to increase the promoter activity. Shibui et al. (*27*) chemically synthesized the hybrid promoter, *pac*, consisting of the -35 region of the strong T5 P25 gene promoter and the -10 and operator regions of the *lacUV5* promoter. The *pac* promoter activity was 2.7-fold higher than the *tac* promoter activity.

Aoyama et al. (*28*) investigated the effect of negative supercoiling on a series of synthetic *E. coli* promoters. These promoters carry perfect consensus sequences at the -35 and -10 regions, but with different spacer lengths. The promoter with 17bp spacing showed the highest activity, and the activity steply decreased both sides of the optimal spacing. By introducing negative superhelicity, the activities of those with 16 and 18 bp spacings were remarkably stimulated by supercoiling.

High rates of transcription can also be attained by increasing the number of promoters which transcribe into the target gene. By use of double *tac* promoters, the human cardiodilatin protein has been expressed in high level (*29*).

Use of Effective Terminators

A factor which has not been exploited fully is the use of transcription terminators to minimize the length of the transcript. Ideally, transcription

Table 1
Expression Vectors for *Escherichia coli*

Vector	Size(kb)	Replicon	Promoter	Genetic marker	reference
pPLc236	4.5	pMB1	λP_L	Amp^R	31
pEX2	5.8	pMB1	λP_R	Amp^R	32
pSTP2	3.7	pMB1	P_{trp}	Amp^R Tet^R	33
pOP203-13	4.9	pMB1	P_{lac}	Amp^R Tet^R	34
pAR2019	4.4	pMB1	$P\phi_{10}$	Amp^R	35
pCKSP6	3.0	pMB1	P_{SP6}	Amp^R	36
pGFIB	4.1	pMB1	P_{lpp}	Amp^R	37
pNH8	5.2	pMB1	P_{tac}	Amp^R galK	38
pKK233-2	4.6	pMB1	P_{trc}	Amp^R	39
pRDB8	2.8	pMB1	P_{T4-32}	Amp^R	40
pTA1529	4.2	pMB1	P_{phoA}	Amp^R	41

should be made to terminate shortly after the coding sequence. In some instances, the use of transcription terminators is essential when using strong constitutive promoters to drive expression (*30*).

Nowadays, there are many expression vectors for attaining high-production of target gene proteins in *E. coli* (Table 1). These expression vectors contain strong/controllable promoter and some of them also contain transcription terminators. High-level expression of the gene is achieved by inserting the gene into the cloning site of the expression vector.

Recently, construction of high-expression and promoter-probe vectors have been applied to Coryneform bacteria. Considerble progress in the molecular genetics of Coryneform bacteria has been achieved using recombinant DNA techniques (*42*). Coryneform bacteria have been traditionally used for amino acid production for animal feed supplementation (Lys, Met, Trp) and for food flavoring agents (Glu, Gly) and so on.

Stabilization of mRNA

A further factor that can increase transcription relates to mRNA turnover. Repetitive extragenic palindromic (REP) sequences are highly conserved inverted repeats present in up to 1000 copies on the *E. coli* chromosome. Higgins et al. (*43,44*) have shown both in vivo and in vitro that REP squences can stabilize upstream mRNA by blocking the processive action of 3′→5′ exonucleases. In a number of operons, mRNA stabilization by REP sequences seems to play an important role in the control of gene expression. Evidence that deletion of the REP sequences from the 3′

ends of the *gdh*A and *gly*A genes causes a reduction in synthesis of the *Gdh*A (*45*) and *Gly*A (*46*) proteins, respectively, implies that this is quite a general function.

(3) Optimization of Translation

Increase of Translation Initiation

In bacterial cells, it has been stated that the sequence of the mRNA in the vicinity of the translation initiation codon contains information required for recognition by the ribosome. There is a strong requirement for bp interaction between the region upstream from the initiation codon (Shine Dalgarno (SD) sequence) and 16S rRNA (*47*). The requirement for the SD interaction is evident from an analysis of the sequences of known bacterial RBSs (*48–50*). Computer analysis of sequence homologies has also revealed that there is significant nonrandomness in the sequence around the initiation codons of natural genes (*49,51,52*).

Numerous studies with model genes have begun to reveal the effects of mRNA structure, base composition and sequence on RBS efficiency in *E. coli* (*53–65*). These studies have permitted the formulation of empirical rules about the design of RBSs for the expression of target genes.

The precise location of the SD relative to the initiation codon and the composition of the intervening mRNA can dramatically affect the level of expression. For example, Shepard et al. (*66*) reduced the distance between the SD and the initiation codon of a β-interferon gene from 11 to 6 nucleotides and obtained a 100-fold increase in the level of expression.

Olins et al. (*67*) have presented evidence showing that when the translation efficiency of recombinant genes is far from optimal, it is desirable to replace the original SD sequence. They found an RBS derived from the region upstream from gene 10 of the phage T7 (g10-L), which codes for the phage coat protein (*68*), that dramatically enhanced the expression of a wide variety of target genes from mammalian, plant and bacterial sources.

Optimization of Codon Usages

An artificial tRNA imbalance may be set up if a highly expressing target gene contains a high proportion of infrequently used codons. Although no concrete evidence is available, the tRNA imbalance can cause a potentially serious starvation of the minor tRNA species, hence increasing errors and reducing elongation rates. Optimization of codon usage is therefore important.

(4) Reduction of Protein Degradation

A strain chosen as the host for a recombinant plasmid can greatly influence the levels of expression of a target gene. In many cases, there is no rational explanation for this effect, but in some cases, it is a reflection of protease activity within the cell.

Eight proteases have been detected in wild-type *E. coli* cells and there may be more (*69*). The protease *La* is a major protease in *E. coli,* and its deficient mutants are widely used. Greenberg et al. (*70*) have observed significant enhancement of IL-3 production in the mutant strain.

Bacillus subtilis is known to have two major extracellular proteases, a neutral metalloprotease and an alkaline serum protease. Because of the presence of these proteases, one cannot attain high-production of target enzymes in the culture medium. Uehara et al. (*71*) used the neutral protease deficient mutant and demonstrated that this enzyme was not essential for sporulation. Kawamura and Doi (*72*) described the construction of a double mutant carrying a deletion in the alkaline protease gene and an uncharacterized lesion in the neutral protease gene. The protease activity reduced to 2–4% of the wild-type in the supernatants from the culture. Using this mutant strain, it shall be possible to attain high-yield of secreted proteins into the culture medium.

The classical solution to the protease problem has been to produce fusion polypeptides. This involves the protection of the recombinant protein by whole or part of a host protein molecule fused to the N-terminus of the recombinant protein. Thus β-galactosidase affords considerable protection for low mol wt peptides such as somatostatin (*15*). The advantage of this method is that the host fusion leader has its own SD ensuring successful initiation of translation. The disadvantage is that a fusion polypeptide is produced and if this is not acceptable, methods for converting it to the natural product must be found.

Stabilization of the Expression Plasmid

By maximizing the transcription and translation, the content of the target enzyme can be easily increased by some hundred folds. In practice, there are still serious problems that need to be solved before this approach can be applied on an industrial scale.

Unfortunately, a recombinant plasmid normally puts an additional demand for energy and nutrients on the host cell. That means that a cell without a plasmid will outgrow its counterpart with a plasmid. By random distribution of plasmids to daughter cells, plasmid-free cells are continuously produced. As a result, the cell culture successively loses productivity. Two questions therefore arise:

1. How does one prevent the drop-out of plasmid, and
2. How does one keep plasmid distribution stably inherited.

Following are some possible solutions to these questions.

(1) Methods for Stable Plasmid Inheritance

Use of Antibiotics

The most common method for the selection of plasmid containing cells is the addition of antibiotics to the cultivation broth. With an antibiotic

resistant gene on the plasmid, the plasmid-containing cells will survive. In industrial scale preparations, however, this is an inadequate solution for the following reasons.

1. The antibiotic and its decomposition products may contaminate the cells thus making the purification process more difficult.
2. The antibiotics add, sometimes considerably, to the cost of the medium.
3. The antibiotics may be hazardous for the health of plant operators.
4. The antibiotics may provide problems for the wastewater treatment system.
5. The wastewater will have to be shown to be free of antibiotics.

For these reasons, antibiotic addition may be adequate for laboratory scale experiments but not practical for industrial scale applications.

Use of a Certain Mutant Strain

A system for selection and maintenance of plasmid-containing cells has been developed using a certain mutant host strain.

Rostock and Hershberger (*73*) developed a system which leads to "suicide" of the cells if the recombinant plasmid is lost. This was achieved by construction of a plasmid carrying the λ repressor into the recombinant plasmid cells, thus lysogenic for a λ mutant.

Skogman and Nilsson (*74*) have chosen another method for plasmid stabilization. They have cloned an enzyme in the protein synthesis machinery, the valyl tRNA-synthetase, coded by the gene *val*S. This gene on the recombinant plasmid is indispensable for *val*S^{ts} mutant cell growth.

Miwa et al. (*75*) employed an *Sm*^{d} mutant that cannot grow without *Sm* and cloned a gene that masks the *Sm*^{d} phenotype in the plasmid vector. The *Sm*^{d} host cell carrying the plasmid became *Sm*-independent, the population of plasmid-containing daughter cells can be maintained when cultured in the absence of *Sm*.

In each case, the plasmids were stably inherited and these exhibited better yields. However, because mutation results in cell growth disturbance, and spontaneous reverse mutation may occur, high-yield plasmid-containing daughter cells were not effectively achieved. Therefore, we focus on the following two points;

1. elimination of antibiotics addition, and
2. using wild-type strain as a host cell.

Control of Cultivation Condition

It should not be forgotten that physiology of the host cell can greatly influence the level of expression. The choice of nutrients, the way in which nutrients are supplied to the culture medium and the environmental parameters such as temperature, dissolved oxygen tension, the concentra-

tion of phosphate (*76,77*) and so on are particularly important. A rational fermentation program is essential if high yields are to be obtained. Careful investigations in these points will contribute to plasmid stability in the host cells.

Immobilization of whole living cells was used as another approach to enhance plasmid stability in cultured recombinant bacteria. Nasri et al. (*78,79*) found plasmid to be extremely stable in continuous cultures with immobilized cells within the gel beads. This appears on account of mechanical properties of the gel-bead system that allow only a limited number of cell divisions (*10–16*) to occur in each recombinant cell before the cell escapes from the gel-bead. This limited number of generations is not sufficient for the plasmid-free cells to appear within the cavities.

However, by optimizing culture condition and immobilizing whole cells for plasmid stability, only quasi-stability is acquired. Next, we therefore review plasmid stabilizing function and its application to industrial purposes.

(2) Application of Stabilizing Function of the Plasmid

Mechanism of Active Partitioning

There are at least two general mechanism for plasmid maintenance. The first relies on passive distribution to the daughter cells for plasmid that are maintained in high-copy number (*80*). Low-copy number plasmids such as F plasmid (*81*), P1 prophage (*82*), and R1(*83*) possess a locus responsible for active partitioning and share some general characteristics. We will now focus on the latter case, active partitioning.

F-plasmid

There are three different mechanisms for F-plasmid, besides the control mechanism of replication. Hiraga (*84*) reviewed the first two mechanisms, *sop* and *ccd*, and Loh et al. (*85*) found the third mechanism, *flm*, in the leading region of the F plasmid.

The *sop* region (*sop* stands for stability of plasmid) presumably acts for equipartition of plasmid DNA molecules to daughter cells (*81,84,86–90*). This partition mechanism (Fig. 1) depends on two *trans*-acting genes, *sop*A and *sop*B, and the *cis*-acting DNA site *sop*C of the plasmid. Hiraga et al. (*81,84*) have described a model for the partition mechanism of the F-plasmid. The *sop*C region acts as a specific DNA site necessary for the actual partitioning, interacting with the cellular components consisting of *Sop*B protein and host proteins. *Sop*A protein may also interact directly or indirectly with the *sop*C region, and/or *Sop*A protein may regulate the expression of the *sop*B gene. The expression of the *sop*A gene may be regulated by *Sop*B protein. The hypothetical partition apparatus is assumed to be related to the cell membrane (Fig. 2).

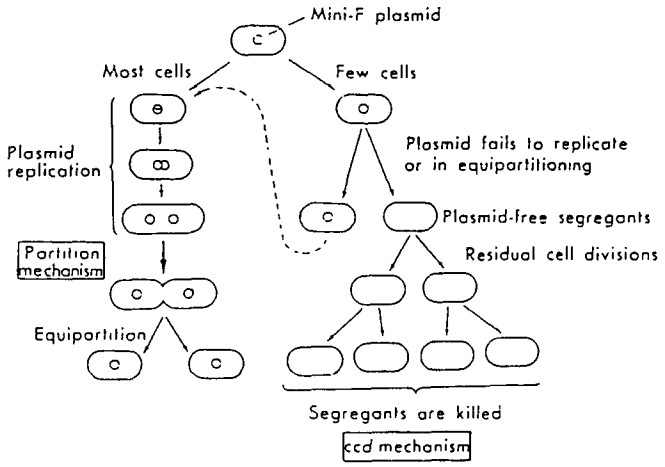

Fig. 1. Scheme showing mechanism of stable inheritance of mini-F plasmid[84] For details, see text.

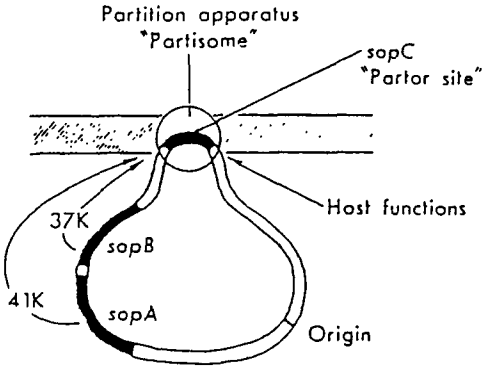

Fig. 2. Hypothetical partition apparatus of mini-F plasmid[84] For details, see text.

Hiraga et al. (*84,91*) also described the second mechanism (Fig. 1) which acts to stably maintain plasmid. This mechanism is specified by two plasmid genes, *ccd*A and *ccd*B (*ccd* stands for coupled cell division). They proposed a nonviable segregant model to explain the functions of *ccd*A and *ccd*B genes when *ccd*$^+$ plasmid replication is blocked (*92*). *Ccd*B protein may inhibit normal cell growth and *Ccd*A protein may suppress the inhibitory function of the *Ccd*B protein. In plasmid-free segregants, these proteins are decreased in concentration and/or *Ccd*A protein is inactivated during residual cell division, so that the *Ccd*B protein inhibits normal cell division via an unknown mechanism. Recently, Feyter et al. (*93*) has presented the autoregulation mechanism of *ccd* operon of F-plasmid.

The third function, *flm* (*flm* stands for *F* leading region *m*aintenance function), may be in the leading region of F-plasmid (*85*). Nucleotide sequencing and functional studies of *flm* locus have shown that it consists

of at least two genes, *flm*A and *flm*B, that are physically and functionally homologous to *hok* and *sok* of *par*B in plasmid R1 (*see below*). The *Flm*A protein is almost identical to the *Hok* protein which has been shown to be a membrane-associated lethal protein (*94*). Gene *flm*B codes for a 100 nucleotide, nontranslated, complementary RNA which over the 5′ leader sequence of the *flm*A RNA. This anti-sense *flm*B RNA binds to the *flm*A RNA and suppresses the expression of the lethal product, presumably by blocking the translation of *flm*A. Secondary structure analysis predicts that *flm*A RNA is extremely stable compared to the regulatory *flm*B RNA. When these RNAs are retained by cells which have lost the F-plasmid, the more stable *flm*A RNA will eventually be translated thus leading to cell death.

P1 Plasmid

The plasmid region responsible for partition of P1 has been isolated and the DNA sequence determined (*95*). Analysis of the sequence showed that the region consisted of two large open reading frames, *par*A and *par*B. Downstream from the open reading frames is a region containing a *cis*-acting recognition site, *par*S, that is essential for partition (*96,97*). It is proposed that this site specifies the attachment of the daughter plasmids to some host cell structure responsible for the selective movement of copies to daughter cells during cell division (*98*) like the case of the *sop* region in the F plasmid.

pSC101

The plasmid pSC101 is not lost from the host cells during one thousand generations under nonselective conditions in spite of its low copy number. Deletion of a 400-bp *par* fragment reduces the plasmid stability. Consequently, the plasmid would be completely lost in less than 100 generations despite the maintenance of its copy number. There is no open reading frame in the essential sequence of the par locus (*99,100*). Rather, two direct repeats and one inverted repeat of 13-bp sequence give it the appearance of a protein binding site. As this sequence can stabilize plasmids unrelated to pSC101 (*101*), it would seem that the sequence provides a binding site for the host protein. Recently, Wahle and Kornberg (*102*) identified a protein in the extract of *E. coli* that specifically binds the *par* locus as DNA gyrase.

R1 Plasmid

The stability locus *par*B of plasmid R1 has been found to specify a unique type of plasmid maintenance function (*103*). Two genes, *hok* (*h*ost *k*illing) and *sok* (*s*uppressor *o*f *k*illing), are required for the stabilizing activity. The *hok* gene encodes a highly toxic gene product, whose overexpression causes a rapid killing and drastic concomitant change in morphology of the host cell. The other gene, *sok*, was found to encode a product that counteracts the *hok* gene-mediated killing. They propose that the

*par*B locus mediates plasmid stability by killing cells that have lost the *par*B+ plasmid during the proceeding cell division, thereby ensuring that a growing bacterial culture predominantly consists of plasmid-containing cells. Another stabilizing locus, *par*A, of plasmid R1 is shown to be localized within 1500-bp region of DNA on the largest *Eco*RI fragment of R1 (*104*). This region has not yet been characterized.

R100 Plasmid

Tsuchimoto and Ohtsubo (*105,106*) cloned the *pem* segment of plasmid R100 containing the two genes, *pem*I and *pem*K (*p*em stands for *p*lasmid *e*mergency *m*aintenance). These genes are responsible for stable maintenance of R100 in dividing cells. The *pem*K gene product functions not by killing the plasmid-free segregant cells, but primarily by inhibiting division of these segregants. Inhibition of cell division secondarily leads to death of the plasmid-free segregants very efficiently.

Other Plasmids

The deletion analysis allowed identification and mapping of the region encoding a partitioning system (*par*) of plasmid RP4, a broad-host range plasmid (*107*). Cloning of this region into several unstable vector plasmid resulted in an increase of segregational stability in all cases. By insertion of the *par* region into an unstable broad-host range mobilizable plasmid and transfer to a series of gram-negative bacteria, it was shown that the cloned par-region of RP4 is functional in a broad-host range.

The *Bacillus* cryptic plasmid pLS11 partitions faithfully during cell division. Using a partition-deficient plasmid vector. Chang et al. (*108*) identified the locus that regulates plasmid partition (*par*) by *cis* complementation in *Bacillus subtilis*. The *par* locus was mapped to a 167-bp segment on pLS11 and 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*. They propose that the pLS11-drived *par* functions as a single-stranded site that interacts with other components involved in plasmid partition during cell division.

Gallie et al. (*109,110*) have mapped and characterized a 5.4 kb region containing the replication origin and stability maintenance of the 44 kb *Agrobacterium tumefaciens* plasmid pTAR.

In general, low copy number plasmids (< 10) have their own way to maintain themselves in cell division. These active partitioning mechanism can be used for industrial-scale cultivation of the recombinant cells. In the following section, we review the application of plasmid partitioning function to make high-stable expression vectors.

Application of Active Partitioning for the Recombinant Plasmid

Gene cloning has evolved as a powerful tool for high-level production of target gene proteins. For these purposes, it is advantageous to develop a system that combines high-level expression (*see above*) and high stability. However, placement of genes on plasmids may result in their instability. The partition locus mentioned previously may be effective in improving plasmid stability.

Boe et al. (*111*) tested plasmid stabilization mediated by *par*A and *par*B genes of the R1 plasmid and *ccd* and *sop* genes of the F plasmid on a mini-R1 plasmid and pBR322 plasmid derivative. The mini-R1 plasmid is thought to be unstably inherited owing to a low copy number and to random segregation of the plasmid at cell division. Cells harboring the pBR322 derivative are lost through competition with plasmid-free cells, mainly as a result of the shorter generation time of cells without plasmids. The insertion of *sop* from F-plasmid or *par*B from R1 plasmid reduced the loss frequency for the pBR322 derivative and the mini-R1 plasmid. Also, insertion of *par*A from the R1 plasmid decreased the loss frequency of the pBR322 derivative and that of the mini-R1 plasmid. When *ccd* from F-plasmid was inserted, the loss frequency of the pBR322 derivative was decreased, but it had only a marginal effect on the stability of the mini-R1 plasmid. In no case was any significant structural plasmid instability observed.

Skogman et al. (*112*) cloned the tryptophan operon of *E. coli* into pBR322 and pACYC184. The partition locus of pSC101 was added to both plasmids, pBR322-trp$^+$ and pACYC184-trp$^+$, to increase stability of the cloning vectors. When the *trp* operon of *E. coli* was cloned in pBR322 and pACYC184, both recombinants became very unstable. The par$^+$ derivatives, pBR322-trp$^+$par$^+$ and pACYC184-trp$^+$par$^+$, were 3- to 10-fold more stable than their *par*$^-$ counterparts in the same experiment, but the partition gene of pSC101 may not be effective enough to obtain full stability.

Nishimura et al. (*113,114*) also used the *par* locus of pSC101 to increase plasmid stability. Recombinant plasmid pYT471, that consists of the aspartase gene (*asp*A) and the high-copy plasmid vector pBR322, was lost from cells of *E. coli* K-12 at high frequency in the medium. This plasmid loss was not completely prevented by the selective pressure of antibiotic addition. Then pNK101 (pBR322-aspApar$^+$) was constructed by using the partition locus derived from pSC101. In *E. coli* cells, pNK101 was lost at a frequency as low as 0.4% per cell generation in nonselective medium. Cells harboring pNK101 produced about 30-fold more aspartate than did cells harboring pYT471 after 30 cell generations. Aspartase production was enhanced by stabilizing the *asp*A recombinant plasmid.

Yukawa et al. (*115,116*) inserted mini-F fragment of F-plasmid into plasmid vector containing the gene for tryptophan synthase, named

Table 2
Stability of the Recombinant Plasmids Containing Partition Locus

Plasmid	Cloned Gene	Stability Locus	Plasmid Stability(%)	Enzyme Activity	Reference
pYT471	aspA	none	10	480	113
pNK101	aspA	par(pSC101)	100	1060	113
pBR322-trpBA	trpB, trpA	none	10	15	116
pBR322F-trpBA	trpB, trpA	sop(miniF)	100	200	116

pBR322F-trpBA. In an *E. coli* K-12 strain cultured in LB broth without selective pressure, pBR322-trpBA was found to be unstable. In contrast, pBR322F-trpBA maintained almost full stability. It was able to be maintained nearly 100% at high copy number in the same culture after 100 generations. The recombinant cells were able to achieve high-level production of tryptophan synthase in the presence of IAA.

The observation that mini-F stabilizes a high copy number plasmid cannot be explained sufficiently by the partition mechanism alone. The fact that pBR322F-trpBA was stably kept at a high copy number in *E. coli* indicates that intensity of replicon on pBR322 is superior to that on mini-F DNA. As a possible explanation, when at least one copy of pBR322F-trpBA is inherited to a daughter cell during cell division, the copy number of that plasmid may be increase by replication on pBR322 in the daughter cell.

Final Comments

In general, high-expression of a target gene leads to plasmid instability. This is especially a problem in large-scale cultivation. However, using the plasmid stabilizing function, like *sop* of F-plasmid, *par* of pSC101, and so on, one can attain both high-expression and high-stability (Table 2). As a result, a continuous high-production is achieved. The major impact of utilizing plasmid partitioning function is on the yield and efficiency of the fermentation process. The application of high-expression and high-stability plasmid mentioned above for other useful bacteria, such as Coryneform bacteria, will be expected in the chemical industry.

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