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Stable Maintenance of Recombinant Plasmid Containing *trp*$^+$ Operon in *E. coli* Cultures by the *pheW*$^+$-*pheS*$^{-ts}$ System

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대장균 배양 중 *pheW*$^+$-*pheS*$^{-ts}$ System에 의한 재조합 *trp*$^+$ 플라스미드의 안정적 유지

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To improve the stability of recombinant pBR322-*trp*$^+$ plasmid (pLTW24) in *E. coli* culture, a positive selection system was devised. A DNA fragment containing *pheW*$^+$ gene (a structural gene for tRNA^{phe}) was isolated and inserted into the pBR322-*trp*$^+$ plasmid (pLTP24). A temperature sensitive host strain, LC901-*pheS*$^{-ts}$, was constructed for this plasmid by transducing *pheS*$^{-ts}$ allele (phenylalanyl-tRNA synthetase) to *E. coli* LC901 using P1*kc* bacteriophage. The LC901-*pheS*$^{-ts}$ cells were unable to grow at a restrictive temperature when they had lost the pBR322 :: *pheW*$^+$-*trp*$^+$ (pLTP24) plasmid. The effects of *pheW*$^+$ gene on the plasmid stability and the expression level of *trp*$^+$ gene in LC901-*pheS*$^{-ts}$ strain were investigated. The proportion of Trp$^+$ colonies among LC901-*pheS*$^{-ts}$ strain carrying plasmid pLTP24 was 99%, whereas that of LC901 strain carrying plasmid pLTW24 was 7% at the end of 20 generations. After 100 generations of growth, the strain LC901-*pheS*$^{-ts}$ carrying plasmid pLTP24 showed little loss of plasmids, while the majority of plasmid pLTW24 in LC901 strain were lost in the same period. The activities of tryptophan synthetase (T. Sase) and anthranilate synthetase (A. Sase) in LC901 strain carrying pLTW24 were about 1.2 times and 1.8 times respectively of those in LC901-*pheS*$^{-ts}$ strain carrying pLTP24.

Recently, the recombinant DNA techniques have been applied for the genetic improvement of the tryptophan producing microorganisms (1-3). Imanaka *et al.* (4) and Huh (5) reported that the recombinant plasmids containing the *trp*$^+$ operon became unstable when tryptophan synthetase levels were raised and the stress caused by the overproduction of the *trp*$^+$ operon enzymes have resulted in the disappearance of the entire recombinant plasmids or deletion of the *trp*$^+$ operon from them. Therefore, for successful production of tryptophan using microorganism carrying the recombinant plasmids harboring *trp*$^+$ gene, it must be necessary to look closely at the stability of the recombinant plasmids before they can be used practically.

The stability of a plasmid may be affected by the genetic characteristics of host cells, the copy number of the plasmid, culture condition, and the genes carried on the plasmid (4). Many studies on the stability of the recombinant plasmid and improvement of the plasmid stability have been reported (5-10). However, most of the results have not completely fulfilled the objectives.

An alternative approach to the plasmid stabilization is cloning an essential gene on the plasmid. Skogman *et al.* (11) described a system in which the

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wild type gene for valyl-tRNA synthetase (*valS*) was inserted into the plasmid and transferred into a host strain carrying a temperature sensitive mutation in its chromosomal *valS*$^+$ gene. In this system, cell growth at the nonpermissive temperature was dependent on the plasmid-encoded wild type enzyme and plasmid-free segregants were thereby counter selected. The *valS*$^+$ gene completely stabilized the inheritance of plasmid with only a slight effect on cell growth for at least 150 generations.

In this paper, we describe a system in which the wild type *pheW*$^+$ gene (structural gene for tRNA^{phe}) was inserted into the recombinant *trp*$^+$ plasmid and transferred to the host strain carrying a temperature sensitive mutation in its chromosomal *pheS* gene (phenyl-alanyl-tRNA synthetase). The effects of *pheS*$^{-ts}$-*pheW*$^+$ complementation system on the expression and stability of recombinant plasmid containing *trp*$^+$ operon in *E. coli* are reported.

Materials and Methods

Bacterial strains, bacteriophage and plasmids

As host strains, *E. coli* LC901 (*trpEA2, pheA, tyrA, trpR, tnaA*) (5) and LC901-*pheS*$^{-ts}$ (*trpEA2, pheA, tyrA, trpR, tnaA, pheS*$^{-ts}$) were used. The source of *pheS*$^{-ts}$ gene was *E. coli* NP37 (12). P1 *kc*, a mutant of P1 bacteriophage was used for transducing *pheS*$^{-ts}$ allele from NP37 into LC901 strain. The sources of *trp*$^+$ and *pheW*$^+$ gene were from plasmid pVH5 (ColE1-*trp*$^+$) (3) and pHW2 (pGEM1-*pheW*$^+$) (13), respectively. The recombinant plasmids, pLTW24 (pBR322-*trp*$^+$) and pLTP24 (pBR322 :: *pheW*$^+$-*trp*$^+$) were constructed in this lab and used as test plasmids.

Materials and media

Reagents used in DNA preparation and enzyme assay were purchased from Sigma chemical company and media from Difco Laboratories. Restriction endonucleases and T_4 DNA ligase were purchased from New England Biolabs or KOSKO and used under conditions recommended by the manufacturer. LB (Luria-Bertani) broth and LB agar were used as enriched media and Vogel-Bonner minimal broth and MM agar containing 0.5% glucose were as minimal media (14). Antibiotics such as ampicillin (Ap) and tetracycline (Tc) were added at the final concentration of 50 and 10 μg/m*l*, respectively. Amino acids, L-tryptophan (Trp), L-phenylalanine (Phe) and L-tyrosine (Tyr) were added to the minimal media at 20 μg/m*l* when needed.

Plasmid preparation, manipulation and transformation

Plasmids were prepared by the rapid alkaline extraction method of Birnboim and Doly (15) and further purified by CsCl-EtBr density gradients centrifugation (16). DNA fragments generated by endonuclease degestion were analyzed by agarose gel elctrophoresis in 89 mM Tris- Cl-borate buffer (pH 8.0). The interested DNA bands were recovered by a dialysis membrane method of Girvitz *et al.* (17). The procedure for transformation followed the method of Nogard *et al.* (18).

Transduction

Tn10 insertion into *E. coli* temperature sensitive mutant strain, NP37, was followed by the method described in Advanced Bacterial Genetics (19). Preparation of P1*kc* (NP37::Tn10) lysate and transduction was by the methods described in Manual of Methods for General Bacteriology (20).

Phenotypic stability of plasmid

After each 20 generations of growth, cells grown in MM broth supplemented with the required amino acids were diluted and spread on LB agar plates. After overnight incubation, 100 colonies were tested for the presence of drug resistance and Trp$^+$ phenotype by transferring them with toothpicks to the selective agar plates (LB + Ap and MM + Phe + Tyr). Phenotypic stabilities of plasmids were determined by the percentage of growth on each selective plate.

Enzyme assay

Tryptophan synthetase activity in the crude extract of cultured cells was assayed by the method of Smith and Yanofsky (21) and anthranilate synthetase activity by the method of Tamin *et al.* (22). The protein content was measured by the method of Lowry *et al.* (23).

Results and Discussion

Construction of plasmids

Plasmid pHW2 DNA used as source for the

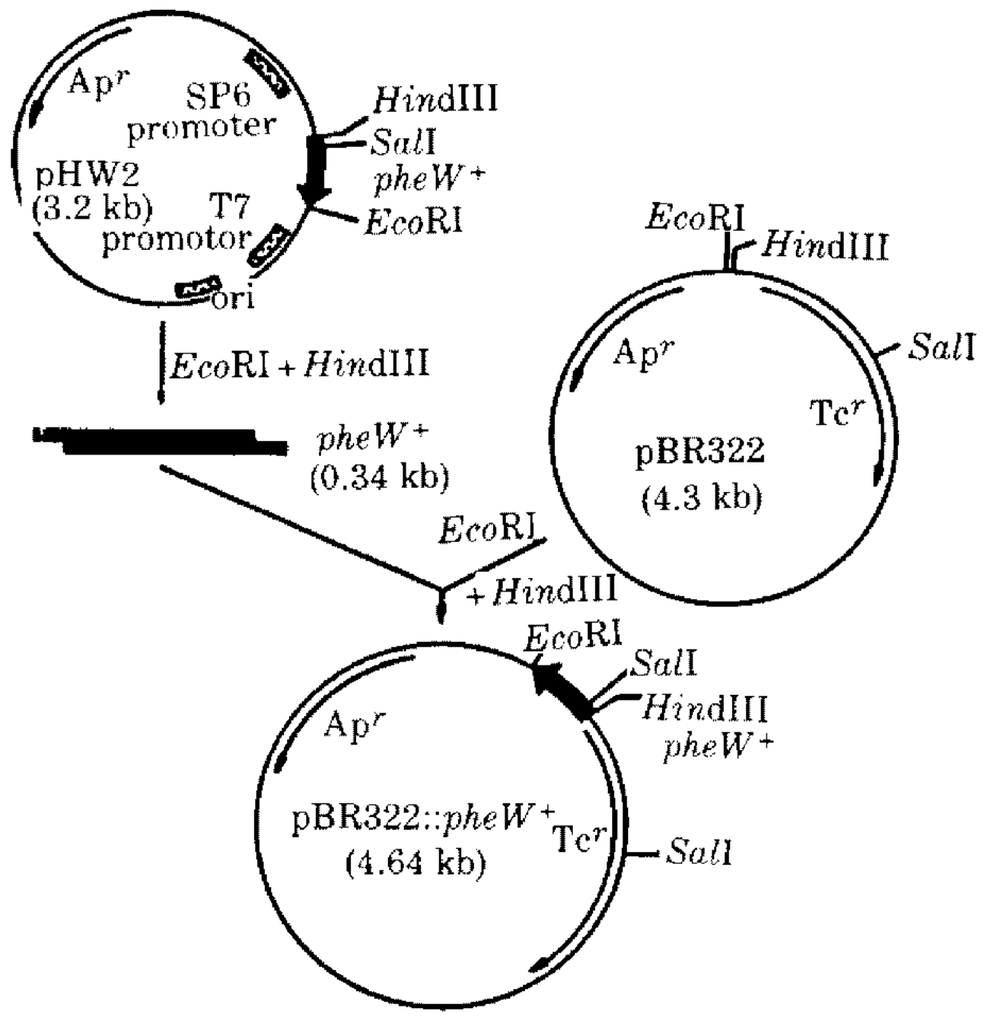

Fig. 1. Schematic procedures for construction of the pBR322-*pheW*+ plasmid DNA.

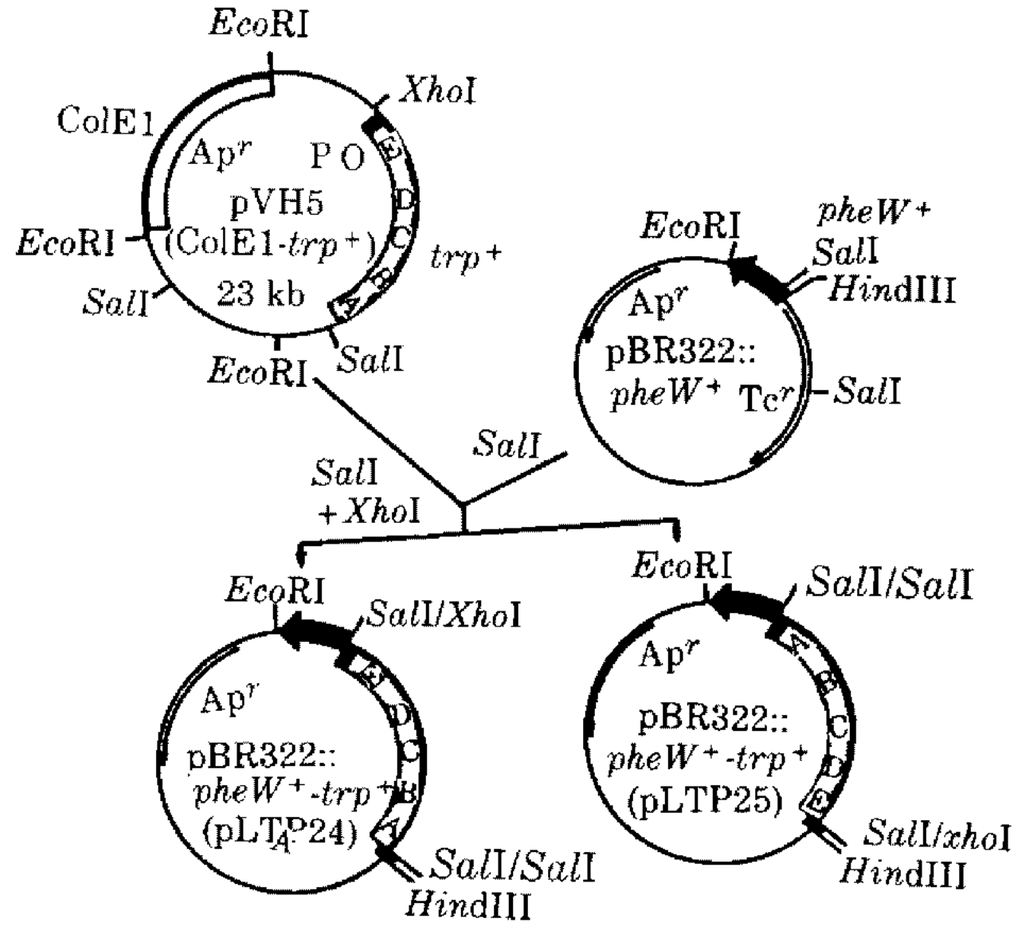

Fig. 2. Schematic procedures for construction of the pBR322::*pheW*+-*trp*+ plasmid DNAs, pLTP24 and pLTP25.

pheW+ gene was digested with *Eco*RI and *Hin*dIII and 0.3 kb DNA fragment was recovered from agarose gel. This *pheW*+ gene fragment was ligated with *Eco*RI and *Hin*dIII-digested pBR322 DNA (Fig. 1) and the ligation mixture was transferred into competent cells of *E. coli* NP37. The transformants were selected on LBAp plate (LB agar containing ampicillin, 50 μg/m*l*) incubated at 41°C. The presence of the plasmid DNA in the colonies with the right phenotype was verified by agarose gel electrophoresis.

For the cloning of *trp*+ gene, plasmid pBR322::*pheW*+ obtained was cleaved partially with *Sal*I and ligated with *Sal*I plus *Xho*I-cleaved pVH5 DNA containing *trp*+ operon (Fig. 2). The recombinant pBR322::*pheW*+-*trp*+ plasmids conferring the right phenotypes of *pheW*+ and *trp*+ gene were isolated. The plasmids obtained have the tryptophan operon in both orientation and the size of 12.1 kb. They were designated as plasmid pLTP24 and pLTP25 (Fig. 2).

Table 1. Confirmation of *pheS*$^{-ts}$ mutant by *pheS*$^{-ts}$-*pheW*+ complementation test.

Strains/Plasmids	30°C	41°C
E. coli NP37	+	−
E. coli NP37/pHW2	+	+
E. coli LC901	+	+
E. coli LC901/pLTW24	+	+
E. coli LC901-*pheS*$^{-ts}$	+	−
E. coli LC901-*pheS*$^{-ts}$/pLTW24	+	−
E. coli LC901-*pheS*$^{-ts}$/pLTP24	+	+

The temperature sensitive mutant, LC901-*pheS*$^{-ts}$, was selected by transferring the transduced cells onto two MM agar plates (VB + Trp + Phe + Tyr) and incubated one each at 30°C and 41°C. Trp phenotypic complementation was confirmed by introducing pLTP24 plasmid into LC901-*pheS*$^{-ts}$ on the selective agar plate (MM + Phe + Tyr). Symbols; +, Growth; −, No growth.

Construction of host strain, *E. coli* LC901-*pheS*$^{-ts}$

To introduce *pheS*$^{-ts}$ allele into *E. coli* LC901 recipient strain, transduction was carried out using P1*kc* generalized transducing phage by the methods described in Material and Methods. After enrichment with ampicillin (100 μg/m*l*) and cycloserine (100 μg/m*l*) in MM broth containing Trp, Phe and Tyr, the temperature sensitive mutant, LC901-*pheS*$^{-ts}$, was selected by transferring the transduced cells onto two MM agar plates and incubated one each at 30 and 41°C. The *pheS*$^{-ts}$ transductant obtained was finally confirmed by *pheS*$^{-ts}$-*pheW*+ complementation test (Table 1).

Phenotypic stability of the recombinant plasmids

The recombinant plasmids pLTP24 (pBR322::*pheW*+-*trp*+) and pLTW24 (pBR322-*trp*+) were transferred into LC901-*pheS*$^{-ts}$ and LC901 host strain respectively. These two transformants have

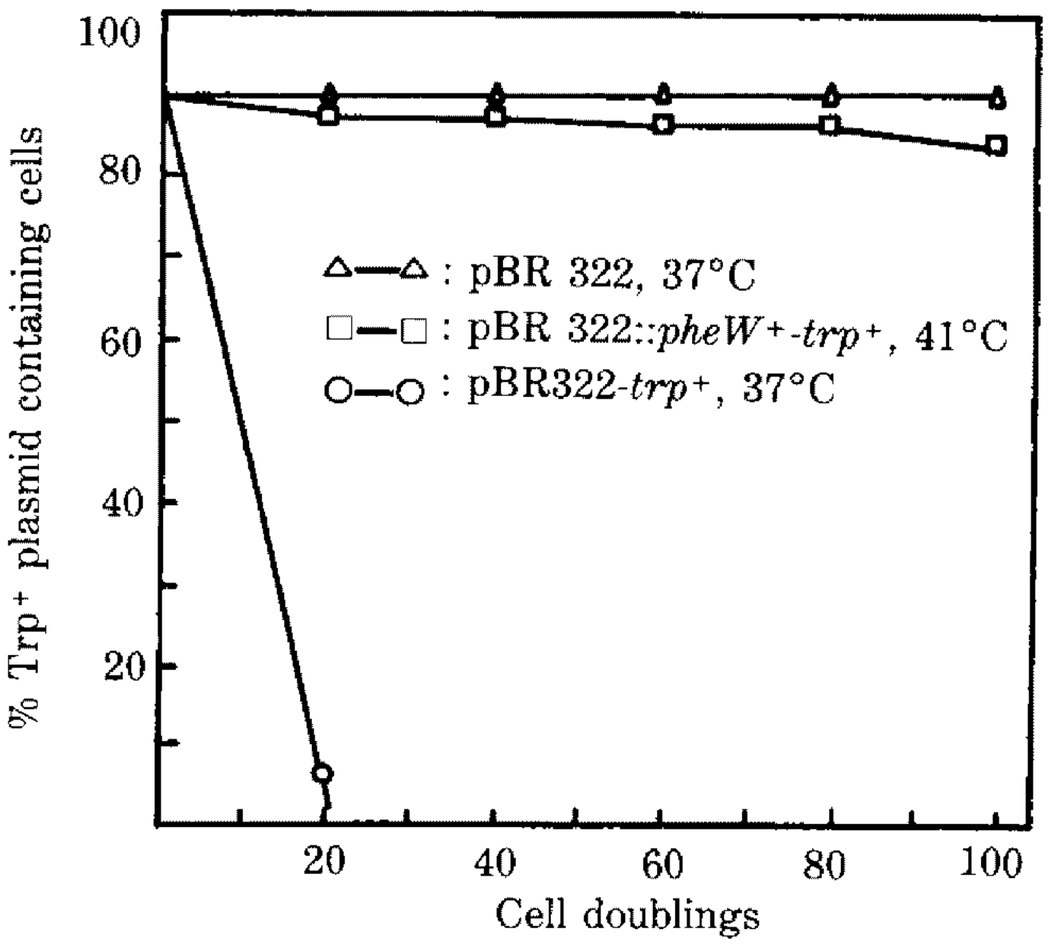

Fig. 3. Phenotypic stability of plasmids, pBR322, pBR322-*trp*$^+$ and pBR322::*pheW*$^+$-*trp*$^+$ during 100 generations.

Cells harbouring the recombinant pBR322-*trp*$^+$ plasmid and pBR322::*pheW*$^+$-*trp*$^+$ plasmid were cultured in VB minimal media at the temperatures as shown. During 100 generations of growth, phenotypic stabilities of plasmid pBR322, pLTW24 and pLTP24 were determined.

shown little difference in the specific growth rate and duration of lag period (data not shown).

After 20 generations of growth, phenotypic stabilities of the plasmid pLTW24 and pLTP24 were shown in Fig. 3. The proportion of *Trp*$^+$ colonies among strain LC901-*pheS*$^{-ts}$ carrying plasmid pLTP24 was 99%, whereas that of strain LC901 carrying plasmid pLTW24 was 7%. The LC901 carrying plasmid pLTW24 showed a very high frequency of plasmid loss from the beginning.

After 40 generations of growth, the majority of the LC901 cells carrying pLTW24 were plasmid free. However, the LC901-*pheS*$^{-ts}$ cells carrying pLTP24 showed little loss of plasmid during 100 generations (Fig. 3) No segregation of the *PheW*$^+$, *Trp*$^+$ and *Ap*^{r} phenotypes was observed in the colonies tested during the same period. For the LC901 cells carrying pLTW24 plasmid, the frequency of plasmid loss increased with time, indicating that the plasmid free segregants may have a growth advantage over plasmid containing cells. In all experiments, a coordinated loss of the antibiotic resistance and tryptophan synthesis markers was found. One explanation for this could be the deletion of whole plasmid due to the energetic burden on the cells as

Table 2. Tryptophan synthetase, Anthranilate synthetase activities and phenotypic stability of the recombinant *trp*$^+$ plasmids, pLTW24 and pLTP24.

	T. Sase (U/mg protein)	A. Sase (U/mg protein)	Plasmid stability (%)
pBR322-*trp*$^+$/ LC901	860	66	7
pBR322::*pheW*$^+$ -*trp*$^+$/LC901-*pheS*$^{-ts}$	699	36	99

The strain was cultured in VB minimal medium at 30°C for pLTW24 harbouring cell and at 41°C for pLTP24 harbouring cell for 20 generations. T. Sase: tryptophan synthetase. A. Sase: anthranilate synthetase.

a consequence of increased plasmid content and gene expression (4, 24). Or an increase in plasmid size, the superhelical density or changes in other physical parameters of the DNA might adversely affect plasmid replication (25).

The relationship between the enzyme activity and the phenotypic stability

As shown in Table 2, tryptophan synthetase (T. Sase) and anthranilate synthetase (A. Sase) activities in LC901 carrying plasmid pLTW24 were about 1.23 and 1.83 times, respectively, of those in LC901-*pheS*$^{-ts}$ strain carrying plasmid pLTP24. In spite of the high frequency of plasmid loss in the LC901 cells carrying plasmid pLTW24, the higher levels of *trp*$^+$ operon enzymes might have resulted from the increased initial expression of *trp*$^+$ operon in the LC901 carrying plasmid pLTW24. Although cells deprived of the *trp*$^+$ genes could not grow in MM, the cells carrying plasmid pLTW24 must have resulted in crossfeeding tryptophan into cells lacking *trp*$^+$ genes.

In LC901-*pheS*$^{-ts}$ strain carrying plasmid pLTP24, growth of the cell at the restrictive temperature must depend on a large amount of plasmid-encoded tRNA^{phe} and thus the presence of the recombinant plasmid became essential to the cells. However, the competition between *pheW*$^+$ gene and *trp*$^+$ gene in transcription may possibly resulted in the decreased synthesis of *trp*$^+$ operon-encoded enzymes.

We have shown here that the *pheS*$^{-ts}$-*pheW*$^+$ complementation system can stably maintain the

recombinant pBR322-*trp*$^+$ plasmid. This system can be also applicable to other *E. coli* host-vector system and thereby be of considerable advantage for industrial fermentation with the genetically engineered bacteria.

요 약

재조합 pBR322-*trp*$^+$ 플라스미드의 숙주내 안정적 유지를 목적으로 tRNA^{phe}의 구조유전자인 *pheW*$^+$ 유전자를 pBR322-*trp*$^+$ 플라스미드에 도입시키고, 숙주세포로는 트립토판 생산을 위한 정상숙주 LC901의 phenylalanyl tRNA synthetase 온도감수성 변이체인 LC901-*pheS*$^{-ts}$를 구성하여 이 온도감수성 숙주의 제한온도(restrictive temperature)에서 재조합 *trp*$^+$ 플라스미드의 안정적 유지와 *trp*$^+$ 유전자와 발현 정도에 *pheW*$^+$ 유전자가 미치는 효과를 조사하였다.

20세대 후, LC901-*pheS*$^{-ts}$ 균주내의 pLTP24 플라스미드는 99%가 보존된 반면 LC901 균주내의 pLTW24 플라스미드는 단지 7%만이 남아 있었다. LC901의 pLTW24 플라스미드의 경우 40세대 후에는 플라스미드가 전혀 남아 있지 않았으나, LC901-*pheS*$^{-ts}$의 pLTP24 플라스미드는 100세대 후에도 거의 안정하게 유지되었다. LC901 숙주내의 pLTW24 플라스미드에 의해 발현되는 tryptophan synthetase와 anthranilate synthetase의 활성도는 LC901-*pheS*$^{-ts}$내의 pLTP24 플라스미드 것보다 각각 1.2와 1.8배로 높게 나타났다.

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