Rapid Paper

Stabilization by the Mini-F Fragment
of a pBR322 Derivative Bearing
the Tryptophan Operon
in *Escherichia coli*Hideaki YUKAWA, Yasuou KURUSU,
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In an *Escherichia coli* K-12 strain (*trpA trpE tna*) cultured in LB broth without selective pressure, a pBR322 derivative bearing the *E. coli* tryptophan operon (pBR322-*trp*) was rapidly lost: after 27 cell-number doublings, only 7% cells retained both tryptophan prototrophy (Trp^+) and ampicillin resistance (Ap^r), and 17% were Ap^r but Trp^- . Insertion of the mini-F DNA from F factor into this plasmid effectively suppressed both the plasmid loss and the discoordinate loss of Trp^+ : the percentage of Trp^- cells per cell-number doubling was decreased more than 100-fold. Partial derepression of the *trp* operon due to 3-indole acrylic acid further decreased the stability of the pBR322-*trp* but not that of the mini-F-inserted pBR322-*trp*.

Expression of the tryptophan (*trp*) operon in *Escherichia coli* must be finely regulated,¹⁾ and tryptophan overproduced on excess expression caused either by *trpR* mutation or by artificial gene amplification results in the inhibition of cell growth.^{2,3)} Thus, high copy number plasmids of the random partitioning type that are defective in plasmid-monomerizing functions, such as pBR322,⁴⁾ are, upon insertion of the *trp* operon, rapidly lost from the host cells cultured without selective pressures.³⁾ In addition to the loss of whole plasmids, partial defects in plasmids rendering the cells tryp-

tophan auxotrophic frequently occurs, especially in *trpR* or *tna* mutants. These properties make it difficult to use *trp* operon-bearing plasmids for industrial production of tryptophan or tryptophan synthase.

Several attempts have been made to overcome these difficulties. Insertion of the partition locus, *par*, of plasmid pSC101 increased the stability of pBR322-*trp* and pACYC184-*trp*,⁵⁾ and plasmids bearing the wild-type *valS* gene were well maintained in a temperature-sensitive *valS* mutant at 37°C.⁶⁾ The stabilization effects found in these studies are however, difficult to evaluate in terms of practical applications, since they did not examine the effect of derepression, and they employed a *tna*⁺ (tryptophanase-containing) strain as the host, in which the tryptophan level tends to be lowered which favors plasmid maintenance.

The stable characteristics of *E. coli* F factor are well known, and the mini-F region, a 9.1 kb EcoRI fragment therefrom, contains all essential functions for its stable inheritance.⁷⁾ In this study, we report satisfactory stabilization of a pBR322 derivative bearing the *trp* operon by the inserted mini-F fragment. Both the loss of whole plasmids and the partial defects causing Trp^- were effectively suppressed by mini-F even with substantial derepression of the operon.

MATERIALS AND METHODS

E. coli K-12 strain JK268 (F^- *trpE61 trpA62 tna-5 purB58 dadR1*, *E. coli* Genetic Stock Center, Yale University) was used as the host for various *trp* operon-bearing plasmids. Cells were cultured either in minimal medium MM⁸⁾ (containing 0.2% glucose and supplemented with 50 μg adenine per ml) or broth medium LB⁹⁾ (supplemented with 50 μg adenine per ml and 0.1% glucose) at 37°C with constant shaking. Plates contained 1.5% agar. Ap was added to selective plates at the concentration of 50 μg per ml. Cell concentrations were determined by counting the colonies formed on LB plates at 37°C.

Plasmids were constructed as depicted in Fig. 1. *E. coli* cells were transformed with plasmids by the method of

Abbreviations: Ap, ampicillin; IA, 3-indole acrylic acid; kb, kilobase pairs; MM, minimal medium supplemented with adenine; r(superscript), resistant; s(superscript), sensitive; Trp^+ , tryptophan prototrophy; Trp^- , tryptophan auxotrophy.

Fig 1. Construction of a *trp* Operon-bearing Plasmid.

Mini-F-*trp* was obtained by ligating the 9.1 kb *Eco*RI fragment of F factor DNA and the 17.4 kb *Eco*RI fragment containing the *trp* operon from ϕ 80*trp* DNA, which was prepared by the method of Maniatis *et al.* This plasmid was digested with *Xho*I and *Sal*I, and the resulting fragments were ligated to the *Sal*I-digested pBR322 to form pBR322-*trp*. pBR322F-*trp* was constructed by inserting the mini-F fragment prepared by digesting Mini-F-*trp* with *Eco*RI into the *Eco*RI site of pBR322-*trp*. The reaction conditions for the restriction endonucleases and T4 DNA ligase were those reported by Maniatis *et al.*¹¹⁾ The molecular sizes of the constructed plasmids and DNA fragments were determined by electrophoresis in horizontal agarose slab gels with a buffer containing 20 mM Na-acetate, 1 mM ethylenediamine tetraacetate and 33 mM Tris-HCl (pH 7.8). The direction of the insertion in each step was examined by digesting the plasmids with appropriate restriction endonucleases and then determining the sizes of the fragments formed. Filled region, the mini-F segment; hatched region, the *trp* operon including segment; open region, ϕ 80*trp* derived segment not including the *trp* operon; fine line, pBR322 derived segment.

Cohen *et al.*,¹⁰⁾ phage DNA was isolated according to Maniatis *et al.*,¹¹⁾ and plasmid DNA was prepared by the cleared lysate method¹²⁾ and by CsCl-ethidium bromide density gradient centrifugation.¹³⁾

For the tryptophan synthase assay, cells harboring various plasmids were precultured overnight in MM, and then cultured in LB medium at 37°C for 8 generations. Preparation of crude cell extracts and the enzyme assay with indole and L-serine as substrates were performed according to the procedures of Smith and Yanofsky.¹⁴⁾ One unit of activity was expressed as the conversion of 0.1 μ mol of indole to tryptophan in 20 min at 37°C.

RESULTS

The phenotypic stabilities of three *trp* operon-bearing plasmids in *E. coli* strain JK268 (*trpE trpA tna*) under nonselective conditions are presented in Table I. pBR322-*trp* was unstable, rapidly yielding cells lacking the plasmid as well as cells with defective plasmids, as reflected by the appearance of $\text{Trp}^- \text{Ap}^s$ and $\text{Trp}^- \text{Ap}^r$ cells, respectively. Addition of IA, an efficient competitive inhibitor of the repressor, and tryptophanyl-tRNA synthetase¹⁵⁾ to

TABLE I. STABILITY OF PLASMIDS BEARING THE *trp* OPERON IN *E. coli* STRAIN JK268^a

Plasmid	Number of ave. gen. ^b	IA ^c	Phenotype distribution (%)				%Trp ⁻ cells formed per ave. gen. ^b
			Trp ⁺ Ap ^r	Trp ⁺ Ap ^s	Trp ⁻ Ap ^r	Trp ⁻ Ap ^s	
pBR322- <i>trp</i>	26.6	—	7	0	17	76	9.5
	23.4	+	2	0	21	77	15.4
MiniF- <i>trp</i>	30.0	—		98		2	0.07
pBR322F- <i>trp</i>	26.0	—	98	0	0	2	0.08
	24.1	+	97	0	0	3	0.13

^a Cells of strain JK268 (*trpA trpE tna*) harboring the plasmids indicated, which were grown in MM to the stationary phase, were inoculated into LB at *ca.* 50 cells per 100 ml, grown at 37°C for the average generations (see below) indicated, and then plated on LB plates with appropriate serial dilutions. After overnight incubation at 37°C, 100 colonies from each plate were examined as to their phenotypes by transferring them with tooth picks to selective plates. Cell concentrations were determined before and after the above cultivation. Experiments repeated at least 3 times gave essentially the same results.

^b ave. gen.: average generation, the time required for doubling of the total cell number in a culture.

^c + indicates that IA was added to LB medium at 100 µg per ml.

the medium enhanced the instability.

To examine the applicability of the stable characteristics of *E. coli* F factor for the stabilization of *trp* operon-bearing plasmids, mini-F-*trp* was constructed (Fig. 1). This plasmid was stably maintained in strain JK268 under nonselective conditions (Table I, line 3). The mini-F fragment was then inserted into the pBR322-*trp* to form a new plasmid, pBR322F-*trp* (Fig. 1). This composite plasmid was also maintained stably. Not only the simultaneous loss of Trp⁺ and Ap^r, but also the segregation of the prototrophy and resistance was effectively suppressed (Table I, line 4). The Trp⁻ cells appearing per cell-number doubling in the culture decreased 135-fold. This plasmid was also maintained stably in cells partially derepressed with IA (Table I, last line).

Expression of the *trp* operon was assessed by measuring the *in vitro* tryptophan synthase activity after cultivation for 8 generations. Specific activities of the cells harboring pBR322-*trp* and pBR322F-*trp* in LB medium were 6 and 16 units per mg of protein, respectively, whereas those in LB containing 100 µg of IA per ml were 41 and 150, respectively. The last value, 150, is equivalent to the maximum value obtained by Imanaka *et al.*³⁾ with a *trpAE trpR tna* mutant harboring

pSC101-*trp*, in which the plasmid was very unstable.

DISCUSSION

The stability of a plasmid in a cell depends on many factors. They include the copy number and active partitioning functions. In the case of *trp* operon-bearing plasmids, a higher *trp* gene dosage and derepression caused by either *trpR* mutation or inhibitors, such as IA, lead to higher expression causing higher production of the enzymes of the operon. These features, desirable for the industrial production of tryptophan or tryptophan synthase, are, however, disadvantageous for the host cells, and slower growth of cells results in the rapid concentration of *trp*-free cells. Another industrially desirable feature, a *tna* mutation, further enhances the plasmid instability.³⁾

The present result that the inserted mini-F fragment stabilized an otherwise unstable pBR322-*trp* even in a *tna* mutant in the presence of IA (Table I) shows the a prospect of industrial application of *trp* operon-bearing plasmids. In the mini-F plasmid, two distinct regions have been shown to confer the stability: a 3.0 kb region including the *sopA*, *sopB* and *sopC* genes that most likely specify the partitioning,¹⁶⁾ and the 0.7 kb *ccd* region that

couples host cell division to plasmid proliferation.¹⁷⁾ Miki *et al.*¹⁸⁾ also obtained results consistent with these notions. It is not clear whether the stabilizing effect of the mini-F observed in the present work is solely due to these functions. The mini-F effectively suppressed not only the loss of whole plasmids, which is easy to undersand in terms of the active partitioning by the above known functions, but also the partial defects in plasmid, as reflected by the discoordinate loss of Trp⁺.

It is very interesting that no deletion of the *trp* operon from pBR322F-*trp* occurred. We believe that the method presented in this paper is effective for stabilization of recombinant plasmids.

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