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DISRUPTION OF TARGETED GENE IN BACTERIAL CHROMOSOME BY USING A TEMPERATURE-SENSITIVE PLASMID

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SUMMARY: The temperature-sensitive plasmid, pSAK3, was used in tryptophan-producing strains for tryptophanase gene disruption to block the degradation of tryptophan. Plasmid pSAK3, which consisted of the pSC103 plasmid containing a tetracycline resistant gene and a disrupted tryptophanase gene inserted by a kanamycin resistant gene, was integrated into the homologous site on the chromosome by gene recombination. Through raising the temperature of cultivation to 42° C and double antibiotics screening process, the strains with disrupted tryptophanase genes could be easily selected and resulted in the generation of stable mutants that no longer exist activity of tryptophanase. Once the strain with a defected tryptophanase gene was obtained, the infection of phage P1 was applied to specifically disrupt the tryptophanase gene of any other strains. This approach also can be applied to disrupt the other targeted genes as an alternative to the conventional mutagenesis. © 1993 Academic Press, Inc.

Conventionally, gene mutation can be done by exposing the cells under chemicals or UV light, and the mutants can be generated by a screening process. There are at least three disadvantages of the random mutagenesis. First, it is impossible to know how the targeted gene was disrupted. Second, not only the targeted gene was destroyed, the other essential genes might also be altered. Third, if the same mutations on various strains need to be performed, the time- and labor-consuming procedure need to be repeated for each strain. Apparently, for a specific purpose, developing a simple and rapid method to disrupt a targeted gene is necessary. Insertion mutagenesis is an alternative to transposon mutagenesis to disrupt a targeted gene of the chromosomal DNA (1, 2), and may avoid the problem encountered in generating recessive mutants by a classical mutagenesis

In this report, we described a new approach of mutagenesis by inserting an antibiotic resistant gene to disrupt the chromosomal targeted gene. A temperature-sensitive plasmid was used in tryptophan-producing *Escherichia coli* for tryptophanase gene disruption. Because the tryptophanase (EC4.2.1.20) catalysis is a major degradation route of tryptophan in *Escherichia coli* during glucose-limiting stage (3), it is of interest to exploit insertion mutant of tryptophanase gene to achieve the improvement of tryptophan productivity. We constructed a plasmid, pSAK3, which consisted of a kanamycin resistant gene flanked by a *Escherichia coli* tryptophanase gene and a temperature-sensitive replicon derived from pSC103 (4), and a tetracycline resistant gene.

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The cells could stably replicate the pSAK3 at 30° C. However, due to the inability of the composite plasmid to replicate at 42° C; most of the cells would not survive in the presence of tetracycline and kanamycin unless the pSAK3 was integrated onto the chromosome. On the basis of the result of double cross recombination, the chromosomal targeted gene would be replaced by a disrupted targeted gene (Fig.1). Therefore, among these integrated strains, the insertion mutant of tryptophanase gene could be selected by picking the kanamycin resistant but not tetracycline resistant strains. It was also very easy to transfer this disrupted gene to the other strains by phage infection, and the mutants could be selected by screening the kanamycin resistant strains. Theoretically, this approach also can be applied to any gene manipulation as long as the complete or partial sequence of the targeted gene is known.

MATERIALS AND METHODS

Microorganisms and Plasmid: *Escherichia coli* DH5a, W3110 Tna⁻, and K12 were obtained from the American Type Culture Collection. The tryptophan-producing strains were previously isolated mutants that contained the feedback resistant *trp operon* screened from 5-fluorotryptophan resistant strains (5). Plasmid, pSC103, a replicon temperature-sensitive plasmid, was obtained from Meacock's laboratory (4). Plasmids pTA73, pTAKn73, and pSAK3 were constructed in this study. The kanamycin resistant gene cartridge plasmid, pUC4-KISS, was purchased from Pharmacia Molecular Biologicals (Sweden).

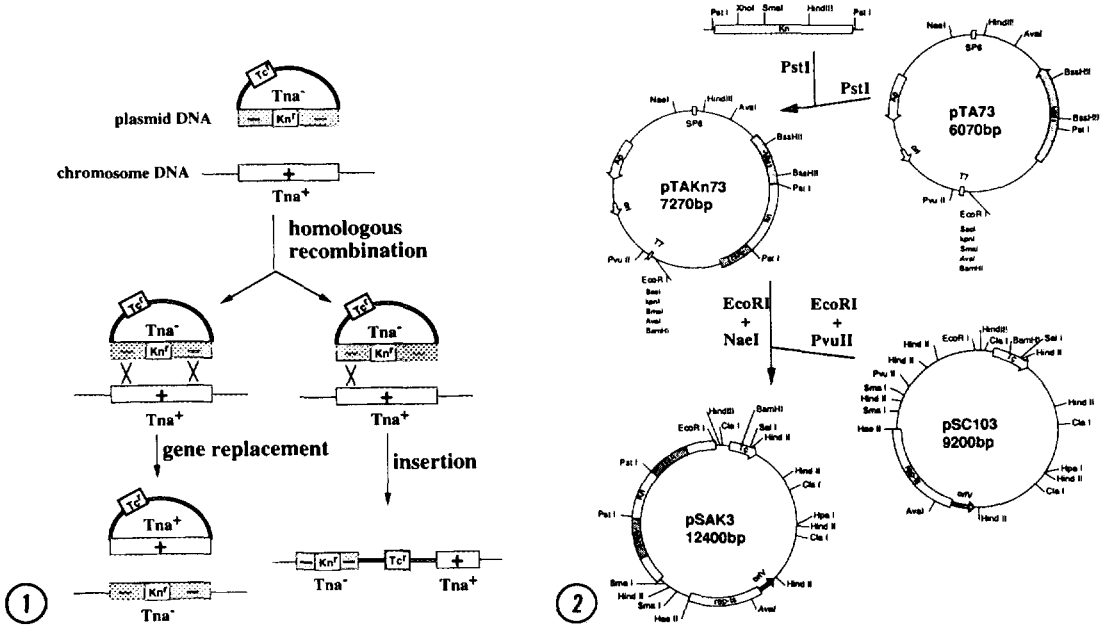

Fig. 1. Schematic representation of the integration events during homologous recombination between plasmid pSAK3 and the *E. coli* K12 chromosome. The double cross-over homologous recombination resulted in gene replacement and the disrupted Tna gene was on the host chromosome. The single cross-over homologous recombination resulted in gene insertion and both the disrupted and normal genes were on the host chromosome. Tna⁻ and Tna⁺ indicate the disrupted and normal tryptophanase genes; Tc^r and Kn^r represent tetracycline and kanamycin resistant genes. [+] and [−] indicate normal and disrupted genes.

Fig. 2. The Construction of Plasmid pSAK3.

Construction of Plasmids : Chromosomal DNA was prepared according to the method of Maniatis et al. (6). Restriction enzymes and T4 DNA ligase were used under the directions provided by the supplier (Boehringer Mannheim Biochemicals, IN, USA). Plasmid isolation was carried out by the alkaline lysis method and purified by CsCl-ethidium bromide equilibrium centrifugation. DNA restriction fragments were recovered from low-gelling-temperature agarose (Bethesda Research Lab., Gaithersbury, USA) and purified as described by Weislander et al. (7). The genomic library of *Escherichia coli* was prepared by using phage EMBL 3 as a vector. A 3.2 kb DNA fragment containing tryptophanase gene could be obtained through restriction digesting and plaque hybridization screening. This DNA fragment was inserted into the BamHI and HindIII cutting site of pGEM3 vector to obtain pTA73. A kanamycin resistant gene from pUC4-KISS could be obtained though PstI digesting, and this DNA fragment was inserted into the PstI site of tryptophanase gene of pTA73 to obtain pTAKn73. Digesting pTAKn73 with EcoRI and NaeI could screen a DNA fragment containing a kanamycin resistant gene and a disrupted tryptophanase gene, which was then inserted into the EcoRI and PvuI site of pSC103 to obtain pSAK3 (Fig. 2).

Disruption of Host Tryptophanase Gene : Plasmid pSAK3 was introduced into *Escherichia coli* k12 as described by Maniatis et al. (6) and the cells were cultivated overnight in the medium containing kanamycin and tetracycline at 30° C. The cells were then spread on the medium plates containing kanamycin, and were cultivated at 42° C. And the colonies resistant to kanamycin but not tetracycline were selected for determining their activities of tryptophanase.

PCR Procedure: The Chromosomal DNA material was added to the PCR mixture to obtain a final volume of 50 µl. PCR mixture consisted of a buffer solution, 50 pmole each of the 5' and 3' primers, Tag DNA polymerase, and 0.1 mM each of dATP, dTTP, dGTP, and dCTP (Boehringer Mannheim). PCR amplification was then carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) in sequential cycles at 94° C for one min, 55° C for 2 min, and 72° C for 3 min with extension step for 20 cycles. The PCR samples were then electrophoresed on 0.8 % agarose gels and the gels washed in water for 30 min to reduce background. Bands on the gel were visualized with 1 µ g/ml ethidium bromide. The chromosomal DNA templates were prepared from *Escherichia coli* K12, tryptophan-producing strains, and plasmids pTA73 and pSAK3. The following two primers were used in this study:

$$\underset{5}{\text{5' - CTTTAAA}}\text{CATCTCCCTGAACC,} \qquad \text{5' - } \underset{464}{\text{GAA}}\text{GTGACGCAATACTTTCG}$$

Enzyme Assay : Tryptophanase (EC 4.2.1.20) was assayed by using a method described by Smith and Yanofsky (8). The unit of activity was the amount of enzyme producing 1 m mole of indole per min at 30° C. Specific activity was given as units of enzyme activity per milligram of protein. The protein concentration was measured according to Lowry et al. (9) using bovine serum albumin as a standard.

Tryptophan Degradation : Cells were previously grown in the LB broth. At mid-logarithmic phase, the seed culture was transferred to 50 ml medium that contained 0.1 % KH_2PO_4, 0.02 % $MgSO_4$, 0.05 % sodium citrate, 0.8 % yeast extract, 7 % glucose, and 3 % $CaCO_3$. The cell growth was carried out at 37° C with constant shaking at 200 rpm in an incubator shaker. To determine the ability of cells to degrade tryptophan, the cells were harvested and resuspended into fermentation medium containing 5 % tryptophan as a sole carbon source. The samples were taken at various intervals for detection of indole, tryptophan and cell density. The cell density was measured at 660 nm by using a spectrophotometer after diluted in water. Tryptophan concentration was measured by a HPLC (Waters Asso.) with an IBM C-18 reverse column (80 % acetonitrile/water mobile system, 1.2 ml/min flow rate) and a UV detector. Indole concentration was measured by mixing equal volume of sample and Ehrlich's reagent (18 g p-dimethylaminobenezaldehyde and 90 ml HCl in 400ml ethanol) and standing for 5 min. The final mixture was then read at 550 nm by a spectrophotometer.

RESULTS AND DISCUSSION

When plasmid pSAK3, carried tetracycline and kanamycin resistant genes as antibiotic markers, was introduced into *E, coli* K12, the cells stably maintained and replicated it at 30° C. However, due to inability of the composite plasmid to replicate at 42° C, most of the cells would not survive in the presence of tetracycline and kanamycin unless plasmid was integrated onto the

host chromosome. The survived strains resulted from the single or double cross gene recombination, and they could be distinguished in terms of the type of antibiotic resistance. Because in pSAK3, a kanamycin but not tetracycline resistant gene was inserted into the tryptophanase gene, strains through double cross recombination had the genotype to resist kanamycin but not tetracycline (Fig. 1), and the tryptophanase gene was replaced by a disrupted gene in those strains. The insertion mutants of *Escherichia coli* K12 no longer had activities of tryptophanase (Table 1).

To obtain the tryptophan-producing strains with defective tryptophanase genes, it took advantage of phage P1 infection to transduct the disrupted tryptophanase gene from insertion mutant to the tryptophan-producing strains. Most of the clonies of phage-infected strains selected from the kanamycin-contained agar plates were no activity of tryptophanase to be detected. The data in Table 1 indicated that the wild type of *Escherichia coli* K12 and the tryptophan-producing strains *E. coli* N25 had very high activities of tryptophanase. The activities of tryptophanase of insertion mutant, *E. coli* K12-Tna$^-$, of the phage-infected mutants of tryptophan-producing strains N25-Tna$^-$, and of W3110-Tna$^-$ strain from random mutagenesis were non-detectable. In a study of tryptophan degradation, the data indicated that the mutants with disrupted tryptophanase genes did not consume tryptophan (Table 1). However, a trace amount of indole was found in the medium, it might form by other pathways but not from tryptophanase-catalyzed route.

To prove tryptophanase gene was disrupted by inserting a kanamycin resistant gene; PCR was used to offer evidence by comparing the molecular sizes of normal tryptophanase gene with the disrupted tryptophanase gene. Plasmids pTA73 and pSAK3 as well as the chromosomal DNA from parental strains and mutants were used as templates, and two known partial sequences from two ends of tryptophanase gene, 5'-CTTTAAACATCTCCCGAACC and 5'-GAAGTGACGC-

Table 1. Tryptophanase Activities of Various *E. coli* Strains and Their Abilities of Tryptophan Degradation and Indole Formation

Strains[c]		Tryptophanase Specific Activity (μmole/min/mg)	Tryptophan[a] Remaining (g/l)	Indole[a] Formation (g/l)
E. coli	K12	2.25	12.5	25.4
	K12-Tna$^-$	ND[b]	49.5	1.5
	N25	3.65	15.0	27.5
	N25-Tna$^-$	ND	48.0	1.0
	G1	2.8	12.0	28.6
	G1-Tna$^-$	ND	50.0	0.5
	W3110-Tna$^-$	ND	42.0	1.9

a. The experiments of tryptophan degradation were performed by transfering the resting cells into the media containing 50 g/l tryptophan, and the tryptophan and indole contents were measured after 16- hour cultivation.
b. ND means non-detectable.
c. Strains N25 and G1 were tryptophan analog-resistant strains (5); strain K12 -Tna$^-$ was obtained in this study by insertion mutagenesis; strains N25-Tna$^-$ and G1-Tna$^-$ were isolated from the phage infection ; strain W3110-Tna$^-$ was obtained by classical mutagenesis.

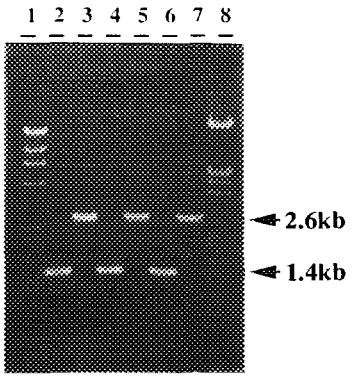

Fig. 3. Agarose gel electrophoresis of PCR gene fragments by various templates. Lanes 1 and 8 : gene markers were from HindIII- and EcoRI-digesting phage λ; Lane 2: plasmid pSAK3, which contained a disrupted tryptophanase gene, was used as a primer; Lane 3 : plasmid pTA73, which contained a normal tryptophanase gene, was used as a primer; Lanes 4-7 : chromosomal DNAs of strains K12-Tna$^-$, K12, N25-Tna$^-$, and N25 were used as primers.

AATACTTTCG were used as primers. After PCR amplification , the DNA fragments were eletrophoresed on agarose gel, and the results shown in Fig. 3 indicated that all the parental strains had the same normal tryptophanase gene fragments of 1.4 kb. On the other hand, all the mutants, in spite of they were derived from insertion mutagenesis or phage infection, had the same disrupted tryptophanase gene fragments of 2.6 kb that contained 1.4 kb fragment of kanamycin resistant gene.

In this study, we demonstrated a system of gene manipulation to specifically disrupt a targeted gene by using a temperature-sensitive plasmid and phage vector. Since the gene recombination was the crucial step of this approach, the cell hosts possessing a recombination mechanism, i.e., strains with rec$^+$ gene must be used. This mutagenesis approach has characteristics of simplicity, quickness, and accuracy, and the screening work is much less than that of classical mutagenesis. This approach can be applied to disrupt any targeted gene as long as the partial sequence of targeted gene is known. Therefore, it is a useful tool to generate special mutants for the study of biochemical pathway.

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