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Cloning and Expression of *Escherichia coli* K-12 *trpL(Δatt) trpE^FBR* gene in *Klebsiella pneumoniae*

Chi. Youn Tae, Ick-Young Kim and Se-Yong Lee
Department of Agricultural Chemistry, College of Agriculture, Korea University, Seoul, Korea
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*Klebsiella pneumoniae*에 있어서의 *Escherichia coli* K-12 *trpL(Δatt) trpE^FBR* 유전자의 클로닝 및 발현

지 연 태 · 김 익 영 · 이 세 영
고려대학교 농과대학 농화학과
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ABSTRACT

A modified *E. coli trp* operon, *trpL(Δatt) trpE^FBR*, was conjugally transfered into *Klebsiella pneumoniae* KC 100 (Phe^-, Tyr^-, Trp^-, Rif^r, Kam^r) by *in vivo* cloning using the hybrid plasmid R_6K:: *Mucts 61* with a transfer frequency of 5.2×10^{-7}. Two *K. pneumoniae* transconjugants, KUA 701 and KUA 702, were isolated. The characters of attenuation control-free and resistance to feedback-inhibition which are characteristics of donor *E. coli trp* operon were normally expressed in the KUA 701. However, KUA 702 retained only the feedback-inhibition resistant character. Trp^+ phenotype and ampicillin resistant character were completely stable in the transconjugants, but streptomycin resistant character was lost in the transconjugants.

INTRODUCTION

Transposable elements are used as genetic tool in the *in vivo* genetic engineering technique (Mendoza *et al.*, 1981). Temperate phage Mu is a member of transposable elements, and inserts linearly its DNA at many different loci in the host DNA. Therefore, Mu seems to be a potentially useful tool for *in vivo* genetic engineering. However, its host range is restricted to certain strains of *E. coli*, *Shigella dysenteriae*, *Citrobacter froundii* and *Klebsiella pneumoniae* (Howe *et al.*, 1975, Rao *et al.*, 1980).

Plasmid R_6K has a broad host range among gram-negative bacteria. Moreover this plasmid is a conjugative plasmid. Most of conjugative plasmids have a low copy number per chromosome. However, the plasmid R_6K has a moderate copy number per chromosome (10~15 copy number/chromosome) (Bukhari *et al.*, 1972). Thus, the hybrid plasmid R_6K::Mu has allowed the introduction of Mu into other strains of gram-negative bacteria. Since R_6K:: Mu can be Hfr form or prime plamid in the host, chromosomal genes can be transfered to the gram-negative bacteria.

Klebsiella pneumoniae (ATCC 25306) has been used for production of chorismate which is a intermediate in biosynthesis of aromatic amino acid. In this study, as an approach

towards breeding of tryptophan-producing bacterial strains, a modified *E. coli trp* operon [*trpL*(*Δatt*)*trpE*^{FBR}] which is not regulated by attenuation or feedback-inhibition with tryptophan, was cloned in *K. pneumoniae* ATCC 25306 by means of the hybrid plasmid R_6K::Mu*cts 61*, and the expression of the *E. coli trp* operon in *K. pneumoniae* was examined.

MATERIALS AND METHODS

Bacterial strains and plasmids used

Bacterial strains used in this study is shown in Table 1.

Media

Penassay broth and LB broth were used for complete medium, and Vogel-Bonner minimal medium was used for minimal medium. Amino-acids added to minimal medium were L-tryptophan (20 μg/ml), L-tyrosine (20 μg/ml), L-phenylalanine (20 μg/ml), L-threonine (20 μg/ml), or L-leucine (20 μg/ml), depending on auxotrophic strains. Thiamine (1 μg/ml), ampicillin (100 μg/ml), streptomycin 50 μg/ml), and rifampicin (200 μg/ml) were added to selection medium when they were required.

Chemicals:

Followings were purchased from Sigma Chemical Company (St. Lewis Mo, USA); DL-5-methyltryptophan, DL-5-fluorotryptophan, Chorismate, and Tris. Bactotryptone, Yeast extract, Casamino acid were purchased from Difco. Other chemicals not mentioned were the first grade or extrapure reagents.

Preparation and titration of phage Mu*cts 61* lysate

A modification method of Razzaki and Bukhari (1975) was used for preparation of Mu*cts 61* lysates of purified lysogens.

Insertion of *Mucts 61* into the R_6K plasmid:

A modification method of Murooka *et al.* (1981) was used for insertion of Mu*cts 61* into the R_6K plasmid.

Matings

Mating was performed on a membrane filter as described by Murooka *et al.* (1981). Donor strains harboring R_6K::Mu*cts 61* and recipient cell, *E. coli* LC 113, were mated on membrane filters (0.45 μm pore size, 25 mm diameter).

Isolation of Trp^+ transconjugants

E. coli LC 113 (R_6K::Mu*cts 61*), a donor cell, was conjugally transfered to *K. pneumo-*

Table 1. Bacterial strains and plasmids used

Strain	Relevant genotype and characteristics	Reference
E. coli		
W 3110	Wild type	
LC 113	*lac tna trpL*(*Δatt*)*trpE*^{FBR}	Y.T. Chi *et al.*
LC 1000(Mu*cts⁻61*)	*thr leu thi*	in this lab.
EG 47	*lac gal hsdR* Mu^s	
E. coli KE 10	*trpC thi* Rif^r Kam^r	in this study
E. coli C	*met thr* Mu^r	
K. pneumoniae		
KC 90	Wild type	ATCC 10031
KC 99	Phe^- Tyr^- Trp^-	ATCC 25306
KC 100	Phe^- Tyr^- Trp^- Rif^r Kam^r	in this study
Plasmid		
E. coli C 600/R_6K	Ap^r Sm^r	

niae KC 100 and *E. coli* KE 10 on membrane filters. Trp^+ transconjugants were selected by spreading on Vogel-Bonner minimal medium containing Ap, Sm and Rif, and reselected in the medium containing 5-methyltryptophan (2×10^{-4}M) and 5-fluorotryptophan (2×10^{-4}M) which are analogs of tryptophan.

Enzyme assay

The anthranilate synthetase was assayed by the method of Tamin *et al.* (1970), and the enzyme activity of tryptophan synthetase was determined according to the method of Smith and Yanofsky (1962). Protein was estimated by the method of Lowry *et al.* (1951).

Stability of recombinant plasmid

For determination of the plasmid stability, cells grown in both LB and the minimal medium containing 20 μg/ml of tryptophan were spreaded on LB agar plate and then the colonies grown on LB agar plate were tooth-picked to both minimal medium without tryptophan and LB containing ampicillin (100 μg/ml) and streptomycin (50 μg/ml).

RESULTS AND DISCUSSIONS

Mu*cts 61* phage yields from lysogen

To determine the phage titers, Mu*cts 61* phage lysate was infected to Mu-sensitive strain, *E. coli* EG 47. The result of phage titers was about 2×10^9 pfu per ml. A phage concentration of 7.8×10^9 pfu/ml could be calculated from thermal induction of *E. coli* LC 113 carring R_6K::Mu*cts 61*.

Transfer frequency of the cloned *trp* operon by R_6K::Mu*cts 61* plasmid

Transfer frequency of R_6K::Mu*cts 61* hybrid plasmid into *E. coli* LC 113 was 2.6×10^{-3}. Table 2 shows the transfer frequency of the cloned *trpL*(Δatt) $trpE^{FBR}$ gene by hybrid plasmid R_6K::Mu*cts*. As shown in Table 1, the transfer frequency was slightly higher than to *K. pneumoniae* KC 100.

Isolation of Trp^+ transconjugants

Two transconjugants, KUE 661 and KUE 662, were obtained from *E. coli* KE 10. From *K. pneumoniae* ATCC 25306, also two transconjugants, KUA 701 and KUA 702, were obtained.

Expression of *E. coli trpL*(Δatt) $trpE^{FBR}$ operon in the Trp^+ transconjugants

Trp^+ transconjugants obtained in this study had *trp* operon originated from *E. coli* LC 113. The genetic characteristics of recombinant *trp* operon in the transconjugants were examined to see whether it is expressed as the same way as in *E. coli* LC 113. Table 3 shows repression of anthranilate synthetase of Trp^+ transconjugants were two fold above wild type. It can be assumed that the increase in the enzyme activity of the transconjugants under derepression condition was caused by increase of trp^+ gene copy number. Anthranilate synthetase activity of transconjugant KUA 701 was higher than the activity of wild type under repression condition. This higher enzyme activity of KUA 701 under repression condition was caused by attenuation-free charactr of the cloned gene. Thus it can be concluded that *E. coli trpL*(Δatt) gene is expressed normally in *K. pneumoniae* as the way it is in *E. coli*. However, anthranilate synthetase activity of transconjugant KUA

Table 2. Transfer frequency of the cloned *trpL*(Δatt)$trpE^{FBR}$ gene by R_6K::Mu*cts 61*

Donor strain	Recipient strain	Selected marker	Transfer frequency*
E. coli LC 113(R_6K::Mu*cts 61*)	*E. coli* KE 10	Ap^r Sm^r Trp^r	6.1×10^{-7}
E. coli LC 113(R_6K::Mu*cts 61*)	*K. pneumoniae* KC 100	Ap^r Sm^r Trp^+	5.2×10^{-7}

* per initial donor cells

Table 3. Repression of anthranilate synthetase in the Trp+ transconjugants

Strain	[Trp]	Specific activity of anthranilate synthetase (units/mg protein)	Relative activity
K. pneumoniae (Wild type)	None	2.1×10^{-2}	100
	10^{-3}M	0.3×10^{-2}	14.3
E. coli LC 113	None	2.2×10^{-2}	104.3
trpL(Δatt)trpE^{FBR}	10^{-3}M	1.9×10^{-2}	90.5
KUA 701	None	4.2×10^{-2}	200
K. pneumoniae(R_6K::Mu*cts 61-trp*$^+$)	10^{-3}M	1.5×10^{-2}	71
KUA 702	None	4.2×10^{-2}	200
K. pneumoniae(R_6K::Mu*cts 61-trp*$^+$)	10^{-3}M	0.5×10^{-2}	24

All strains were grown in VBMM or VBMM plus tryptophan.

Table 4. Repression of tryptophan synthetase in the Trp+ transconjugants

Strain	[Trp]	Specific activity of tryptophan synthetase (units/mg protein)	Relative activity
K. pneumoniae(wild type)	None	4.3	100
	10^{-3}M	0.8	18.6
E. coli LC 113 *trpL(Δatt)trpE*^{FBR}	None	17.3	402.3
	10^{-3}M	14.2	332.5
KUA 701	None	29.2	679.1
K. pneumoniae(R_6K::Mu*cts 61-trp*$^+$)	10^{-3}M	18.6	432.6
KUA 702	None	23.2	539.5
K. pneumoniae(R_6K::Mu*cts 61-trp*$^+$)	10^{-3}M	6.5	151.2

All strains were grown in VBMM or VBMM plus tryptophan.

702 was decreased by 90% under repression condition. This loss of attenuation-free character might be resulted from a rearrangement of *trp* operon or recombination of passenger *trpL(Δatt) trpE*^{FBR} gene with the host *trp* gene.

Table 4 shows tryptophan synthetase activity of transconjugants. Tryptophan synthetase activities of transconjugants under derepression condition were 6~7 times above that of wild type. And, under repression condition, tryptophan synthetase activity of wild type was decreased by 80% in KUA 701, 75% decreased in KUA 702.

These findings also suggest that KUA 701 have the genetic character of *E. coli* LC 113 *trpE(Δatt)*, but not KUA 702. The feedback-inhibition resistance character of the cloned *trp* operon in the transconjugants were also examined. As shown in Table 5, the cloned *trp* operons in the transconjugants have the genetic character which is resistance to feed-back-inhibition by tryptophan.

According to the results of above genetic characteristics of the cloned *trp* operon in the transconjugants could be summerized as shown in Table 6.

Table 5. Feedback inhibition of anthranilate synthetase in the Trp^+ transconjugants

Strain	[Trp] added to mixture	Specific activity of anthranilate synthetase (units per mg protein)	Relative activity
K. pneumoniae(wild type)	None	2.1×10^{-2}	100
	5×10^{-4}M	0.1×10^{-2}	4.76
E. coli LC 113 *trpL(Δatt)trpE*^{FBR}	None	2.2×10^{-2}	104.7
	5×10^{-4}M	1.9×10^{-2}	90.5
KUA 701	None	4.2×10^{-2}	200
K. pneumoniae(R_6K::Mu*cts 61-trp*$^+$)	5×10^{-4}M	1.5×10^{-2}	70.5
KUA 702	None	4.2×10^{-2}	200
K. pneumoniae(R_6K::Mu*cts 61-trp*$^+$)	5×10^{-4}M	2.0×10^{-2}	95.2

Table 6. Genotypes ofthe cloned *E. coli trp* operon in th transconjugants

Transconjugant	Genotype
KUA 701 *K. pneumoniae*(R_6K::Mu*cts 61-trp*$^+$)	*trpL(Δatt)trpE*^{FBR}
KUA 702 *K. pneumoniae*(R_6K::Mu*cts 61-trp*$^+$)	*trpE*^{FBR}

Table 7. Stability of antibiotic resistancy and Trp^+ phenotype in transconjugants

Strain	Culture medium	Growth	Number of colonies		
			Ap^r (100 μg/ml)	Sm^r (20 μg/ml)	Trp^+
KUA 701	LB	+	100	0	100
K. pneumoniae(R_6K::Mu*cts 61-trp*$^+$)	VBMM	+	100	0	100
KUA 702	LB	+	100	0	100
K. pneumoniae(R_6K::Mu*cts 61-trp*$^+$)	VBMM	+	100	0	100

Stability of the R_6K::Mu*cts 61-trp*$^+$ hybrid plasmid

As shown in Table 7, the hybrid was stable in both complete medium and minimal medium. However, transconjugants could not be grown in the medium containing either 50 μg/ml or 20 μg/ml of streptomycin. It may be that the sensitivity was caused by rearrangement of Sm region in the plasmid R_6K. The Trp^+ phenotype was completely stable in the both media.

적 요

트립토판 생산성 증대를 위한 균주 개발의 한 시도로서 transposable element의 일종인 phage Mu와 그람음성세균에 넓은 숙주범위를 갖고 있는 conjugative plasmid인 R_6K를 사용하여 hybrid plasmid인 R_6K:: Mu*cts 61*을 구성하고, 이 hybrid plasmid를 이용하여 트립토판에 의한 feedback inhibition과 attenuator control이 해제된 *Escherichia coli*의 *trp* operon(*trpL(Δatt) trpE*^{FBR})를 *Klebsiella pneumoniae*에 *in vivo* cloning하였다.

R_6K::Mu*cts 61-trp*$^+$ hybrid plasmid를 구성하여 *K. pneumoniae* KC 100에 conjugal transfer하였을 때의

conjugal transfer frequency는 5.2×10^{-7}이었으며, 이때 두 개의 transconjugant KUA 701과 KUA 702를 분리하였다. Transconjugant들의 *trp* operon 산물인 anthranilate synthetase와 tryptophan synthetase 활성을 측정하여 *E. coli*의 *trp* operon이 *K. pneumoniae*에서 어떻게 발현되는지 조사하였다. Transconjugant KUA 701는 공여 *E. coli trp* operon의 유전적 특성이 정상적으로 발현되는 반면, KUA 702는 attenuator control을 받았으나 feedback inhibition에 대해서는 저항성을 갖고 있었다. Transconjugant들의 Trp^+형질과 ampicillin 저항성은 매우 안정하였으나 streptomycin에 대한 저항성을 상실하였다.

이상의 결과로부터 새로이 구성된 hybrid plasmid인 R_6K::Mu*cts 61*을 이용하여 간편하게 *E. coli*의 *trp* operon을 *K. pneumoniae*에 *in vivo* cloning할 수 있었으며, *trp* operon이 *K. pneumoniae*에서 *E. coli*에서와 마찬가지로 발현될 수 있음을 확인하였다.

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