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Cloning and Transcription of *Escherichia coli* Cell Division Gene, *sep*

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E. coli 세포분열 유전자 *sep* 의 Cloning 및 Transcription 에 관한 연구

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ABSTRACT

Sep gene, which is one of the cell division genes coding for penicillin binding protein 3 was subcloned from λ 607*sep*$^{+2}$ to plasmid pBR 322. To maximize the expression of *sep* gene, the *sep* gene was also cloned to plasmid pLJ 3 which has a strong promotor such as *lac* UV 5 (*lac*P). It was confirmed that the *sep* gene cloned to pLJ 3 was in the proper orientation for expression from lactose promotor. To analyze the expression efficiency of *sep* gene within the plasmids newly constructed, *sep* mRNA was assayed by using λ 607*sep*$^{+2}$ DNA as a probe. *Sep* mRNA level was increased 25 times in the cells carrying *sep* gene cloned to pBR 322 compared to *E. coli* C 600 which has wild type *sep* gene within the chromosome instead of plasmid. Furthermore, the cells carrying *sep* gene cloned to pLJ 3 directed the synthesis of about 50 times as much *sep* mRNA as did cells carrying *sep* gene cloned to pBR 322, representing that the *sep* gene was successfully cloned to pLJ3.

INTRODUCTION

Numerous bacterial mutants defective in some aspects of cell division or envelope growth have been described. These include DNA synthesis mutants, mutants with abnormal shape, and altered permeability mutants. However, the search for ts mutants specifically defective in septation has identified only sixteen genes thus far. These mutants continue growth, DNA synthesis, and nuclear region segragation; however, long non-septate filaments form at the non-permissive temperature. Filaments of some of these mutants will divide upon temperature reduction but others have irreversible defects. The mutants have been isolated and mapped by a number of laboratories (Richard *et al.*, 1973; Allen *et al.*, 1972, 1978; Walker *et al.*, 1975) and sequenced (Nakamura, 1983).

The best studied cell division gene is probably *sep/fts* I. It was shown that there was a cluster of cell division related genes near minute two in the sequence: *leu sep murE F C ddl ftsA envA* by transduction heteroduplex analysis, and restriction enzyme analysis (Fletcher *et al.*, 1978; Irwin *et al.*, 1979). *Sep* was shown to code for penicillin binding protein 3 (PBP-3) by transduction analysis of Spratt's PBP-3 ts mutant (selected on the basis of penicillin resistance) (Spratt *et al.*,

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1975) and Hirota's *fis I* mutant (identified by screening ts mutants for PBP-3 activity (Suzuki *et al.*, 1978) and by assay of PBP-3 in *sep* ts mutants (Irwin *et al.*, 1979). Penicillin binding protein 3 can be assayed by its penicillin binding activity although the exact role of PBP-3 in vivo is unknown, it does function specifically in septation. In our study, *sep* gene from λ607 sep^{+2}(C. Irwin, her Ph. D dissertation) has been subcloned to pBR 322 to facilitate molecular studies of *sep* gene. To isolate large quantities of pure penicillin binding protein 3, *sep* gene has been also fused to a strong promotor, such as the *lac*UV 5 promotor (*lac*P) (Roberts *et al.*, 1979). The *sep* mRNA has been assayed in cells having these cloned plasmids by using λ 607 sep^{+2} DNA as a probe.

MATERIALS & METHODS

Strains, plasmids, and phages

The bacterial strains, plasmids, and phages used are given in Table 1.

Media and buffers

Yeast extract tryptone medium (YET) (Howard-Flanders *et al.*, 1964) was supplemented with 0.5% NaCl unless other wise stated. This medium supplemented with 0.001 M $MgSO_4$ was used for lysate preparation. LPGB-CAA, which is 0.1 M Tris-HCl (pH 7.2), 1% glucose, 0.0005% Bl, 1 mM potassium phosphate, 8 mM$(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 1×10^{-6}M $FeSO_4$, 0.03 mM $Ca(NO_3)_2$ (Hirota *et al.*, 1977) was supplemented with 1% Casamino acid. Minimal media (Howard-Flanders, 1964) contained glucose (10 mg/ml), thiamine. HCl (5 μg/ml). Phage suspensions were diluted in λ dilution buffer containing 0.01 M potassium phosphate (pH 7.0) and 0.01 M $MgSO_4$. Cesium chloride (CsCl) buffer (0.05 M Tris. HCl, pH 7.5, 0.1 M NaCl) was used in the preparation of CsCl gradient for phage purification. Restriction endonuclease buffers were: (High salt) 100 mM NaCl, 6 mM $MgCl_2$, 6 mM Tris (pH 7.6), and 2 mM dithiothreitol; (low salt) 6 mM KCl, 10 mM $MgCl_2$, 10 mM Tris (pH 7.6) and 10 mM dithiothreitol.

Table 1. Bacterial strains, plasmids and phages

Bacterial strains	Relevant marker	Genotype/ Phenotype	Source
C600		*thi thr leu*	M. Meselson
2 eolc		F^-*thr leu thi lac rpsi*	M. malamy
AX 655	ts 2158	F^- *thr leu thi arg proA his gal xyl ara mtl lac rpsL*	J. Walker
λimm 434			E. Cox
λ 607 sep^{+2}			C. Irwin
pBR 322	Tet^r Amp^r		
pLJ 3	Tet^r		M. Ptashne

Preparation of phage and plasmid DNA

Double lysogen carrying λimm^{434} and λ 607 sep^{+2} was grown at 30°C to A_{540} of 0.5 (about 5×10^8 cells/ml) in a YET broth and thermally induced at 42°C for 20 minutes. Induced cultures were then incubated at 37°C until lysis was completed. The phages were concentrated by precipitation with polyethylene glycol (Yammamoto *et al.*, 1970) followed by resuspension in λ dilution buffer. Phage suspension was purified in cesium-chloride step gradients with layers of density(ρ) 1.6, 1.5, 1.4, 1.3. These gradients were centrifuged in an SW 41 rotor for 2.5 hr at 30,000 rpm at 4°C. Visible band(s) of phage in the 1.5 ρ region were collected and pooled in an SW 50.1 centrifuge tube. This tube was then filled with 1.5 ρ CsCl solution for an equilibrium centrifugation for 22~24 hr at 35,000 rpm at 4°C. The phage suspensions collected from the equilibrium gradient were dialyzed against phage dialysis buffer (10 mM Tris. HCl, pH 7.5, 10 mM $MgSO_4$). To disrupt phage head, purified phage suspension was

dialyzed against two changes of 50% formamide in 10 mM Tris. HCl, pH 7.3, 1 mM EDTA at room temperature for 90 minutes each. For DNA extraction from the phage, the phage suspension was extracted with equal volume of saturated phenol three times. The aquous layer containing the DNA was then dialyzed into 1 mM EDTA, and the A_{260} of the resulting DNA solution was measured. The two strands of λ 607 sep^{+2} DNA was separated essentially according to Miller (1972). Plasmid DNA for large scale preparation was isolated according to Clewell & Helinski (1969).

Cloning and transformation

The hybrid plasmids were constructed essentially by the procedure of Cameron *et al.* (1975). 1 to 8 μg of each DNA was cleaved by restriction endonuclease (1 to 5 units) at 37°C for 3 hr. The DNA was then heated to 65°C for 3 min. Vector and target DNA were then mixed in ratios of 1 : 8, 1 : 4 or 1 : 2 in 50 to 100 μl of ligation buffer and incubated with 0.5 to 4.0 units of T_4 DNA ligase and 60 μM ATP at 10°C for 18 hr. Transfection with the hybrid plasmids subcloned from λ 607 sep^{+2} was done with strain AX 655 λimm^{434} to prevent lysis of the cells by λ 607 sep^{+2} phages formed during the ligation reaction, according to the procedure of Mandel *et al.* (1970). 20 ml of cells were grown in YET broth at 30°C to A_{540} of 0.3. The cells were centrifuged, resuspended in 10 ml of 0.1 M $MgCl_2$ at 0°C, centrifuged again, resuspended in 0.1 M $CaCl_2$, incubated on ice for 20 min, centrifuged, and finally resusspended in 2 ml of 0.1 M $CaCl_2$ at 0°C. 0.2 ml of these cells were mixed with 0.1 ml of DNA in TES buffer and placed on ice for one hour. The cells were then heat-shocked at 42°C for 1.5 min, 1.7 ml YET broth was added and the cells were incubated with shaking at 30°C for two hr. The cells were then spread on YET plates containing 25 μg/ml tetracycline. Tet^r and TS^+ strains were screened. Other transformation was done with strain AX 655.

Restriction enzyme mapping

The plasmid DNA was prepared from each transformant by the procedure of Birnboim and Doty *et al.* (1979). For restriction enzyme mapping, 1μg DNA was digested in total 20 μl of reaction volume. Electrophoresis was done in horizontal agarose slab gel according to McDonnell *et al.* (1977). Unless otherwise noted, agarose gels were 0.7% or 0.5% agarose. Gels were electrophoresed at 50 or 100 volts for 3 to 48 hr depending on the sizes of the DNA fragment of interest.

Labell ng and isolation of RNA

Cultures were grown in 10 ml LPGB-CAA broth at 37°C to A_{540} of 0.3. Strains containing amplifiable plasmids were then incubated with 150 μg/ml chloramphenicol for 15 hr at 37°C with shaking. The cells were pelleted, washed, and resuspended in LPGB-CAA. After shaking at 37°C for 30 minutes, the cultures were pulse-labelled for 2 minutes with 200 μci H^3-Uridine. The cells were killed with azide, washed and disrupted with 0.5% SDS in 10 mM Sodium acetate, pH 5.0, and 1 mM EDTA. The proteins were extracted twice with phenol. The RNA was ethanol precipitated, centrifuged, and resuspended in TES or 2×SSC.

DNA-RNA hybridization

The filter method of DNA-RNA hybridization was performed by the procedure of Miller (1972). Southern hybridization of RNA to separated strands of phage DNA was also carried out as described by Miller (1972). The DNA was heated at 67°C for 4 hr to renature any complementary strands.

RERULTS & DISCUSSIONS

Cloning of *sep* pBR 322

An Eco RI fragment from one of the defe-

ctive phages cloned into charon 10, was 18.2 Kb and included *leuA*, *sep*, *murE*, *F*. The Sam7 derivatives of this hybrid phage caused the overproduction of PBP-3 after infection (Irwin *et al.*, 1979). To reduce the size of the cloned fragment further, DNA from a charon 10-sep was cut with Eco RI under conditions which reduce specificity. These fragments were ligated to λ 607, which has one Eco RI site, by C. Irwin (her Ph. D dissertation). One hybrid contains a 3.7 kb fragment, bounded by reconstituted Eco RI site, which is enough to code for 120,000 daltons of protein. Since *sep* gene product, penicillin binding protein 3, has a molecualr weight of 60,000 daltons, *sep* constitutes at least 50% of the cloned fragment. If *sep* is transcribed in a precursor form which is then cleaved to generate a mature form, as are some other membrane proteins, the *sep* gene might account for more than 50% of the fragment. This 3.7 kb fragment was cloned into the Eco RI site of plasmid pBR 322 to facilitate transcription and sequencing studies. The hybrid plasmids were screened by their complementing ability of temperature sensitivity of host cells (AX 655). The map and agarose gel electrophoresis of this hybrid plasmid was shown on figure 1 A & 2.

The size of the cloned 3.7 kb fragment was reduced to make it specific for *sep*, generating pNI1 31 (figure 1 B). pNI 18 was partially digested with Pst I, ligated, then transformed to AX655. Tet^r and TS$^+$ strains were selected and the size of plasmid and orientation of *sep* fragment were confirmed. This plasmid contains a 3.0 kb fragment. This fragment is enough DNA to code for 100,000 daltons of protein. The isolation of this plasmid indicates that the *sep* gene must be on the clock-wise side of the Pst I site. The 3.0 kb fragment will not require much further reduction to make it specific for *sep*.

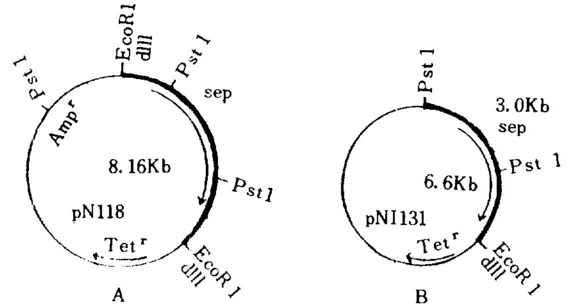

Fig. 1. Restriction map of plasmid pNI 18 and pNI 131. Thin lines are plasmid DNA, thick lines are chromosomal DNA. A. pNI 18 was made by subcloning of 3.7 kb *sep* gene to pBR 322. B. pNI 131 wai made by partial cleavage of pNI 18 by Pst I digestion and religation, arrows represent transcription direction.

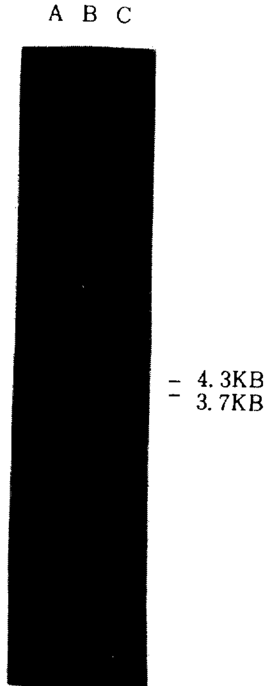

Fig. 2. Agarose gel electrophoresis of pNI 18. pNI 18 was digested with Eco RI, loaded on 0.7% agarose gel. Lane A. Uncut pNI 18. Lane. B pNI 18 digested with Eco RI. Lane C. λ-Hind III, molecular weight standard.

Cloning of sep gene to pLJ 3

To maximize the expression of *sep* gene, 3.7 kb of *sep* gene fragment from pNI 18 was fused to a strong promotor, such as the *lac* UV 5 promotor. The general plan was to

link the 3.7 kb *sep* fragment to *lac*P within pLJ 3 constituted by Ptashne *et al.* (1978, 1979). Specifically, the HindⅢ *sep* fragment from pNI 18 was linked to the Hind Ⅲ site which is adjacent to *lac*P in pLJ 3. pLJ 3 is pMB 9 containing an Eco RI fragment composed of two tandem *lac*P *lac*Shine Delgarno sequences (*lac*SD) and the Hind Ⅲ site only 29 base pairs from the *lac*P insert. pNI 201 is pLJ 3 into which *sep* has been inserted. Based on figure 4, either orientation of pNI 201 could be produced by cloning of 3.7 kb fragment to pLJ 3. If the direction of *sep* gene within plasmid pNI 201 is A, molecular size of 2.0 kb, 1.85 kb, 5.45 kb of fragments should be produced by double digestion with Sal Ⅰ and Hind Ⅲ. Otherwise, if the direction *sep* gene is reverse state like B, molecular size of 2.0 kb, 0.95 kb, 6.53 kb of fragments should be produced. Figure 3 showed the restriction enzyme analysis by Sal Ⅰ & Hind Ⅲ. pNI 201 produced the three fragment of 5.3 kb, 2.0 kb, 1.85 kb indicating that A is the proper orientation for expression from *lac*P.

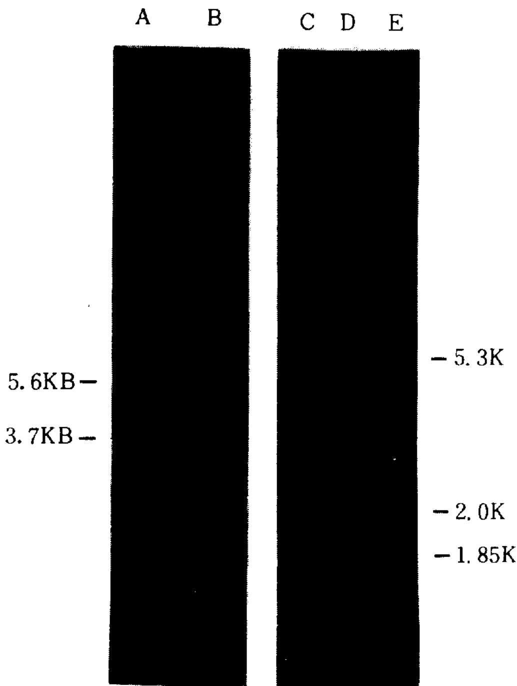

Fig. 3. Agarose gel electrophoresis of pNI 201. pNI 201 was digested with Hind III, analyzed by 0.7% agarose gel. Lane A. pNI 201 cut with Hind III, 3.7 kb fragment represent *sep* gene, 5.6 kbfragment represent vector DNA(pLJ3). Lane B. λ-Hind III, molecular weight marker. Lane C. pNI 201 uncut, Lane D.λ-Hind III & Eco RI, molecular weight marker, Lane E. pNI 201 double digested with Hind III & Sal I.

Analysis of *sep* mRNA synthesis

To test the transcriptional ability of cloned plasmid, the *sep* mRNA was measured by using λ 607 sep^{+2} DNA as a probe. Table 2 shows the hybridization results. Since there are only 50 molecules of penicillin binding protein 3 present per cell (Spratt, 1977), the ability to detect *sep* mRNA was concerned. However, this mRNA constituted about 0.05% of pulse-labelled RNA in wild-type cell such as *E. coli* C600. Presence of hybrid plasmid pNI 18 increased the *sep* mRNA level to 0.75%. The RNA which hybridized to λ 607 sep^{+2} DNA bound mostly to the right DNA strand. from the known orientation of the 3.7 kb insert in this hybrid phage. It could be con-

Table 2. Hybridization of pulse-labelled(H^3) RNA with DNA of λsep^{+2} phages

RNA source	DNA source	% input hybridized[1]
C 600	λ 607 sep^{+2}	0.05±0.01
C 600	λ sep^{+82}	0.09±0.01
pNI 18	λ 607 sep^{+2} (right strand)	1.65±0.05
pNI 18	λ 607 sep^{+2} (left strand)	0.4 ±0.05
pNI 201 Amplified[2]	λ 607 sep^{+2}	47.5 ±0.05
pNI 18	λ 607 sep^{+}	2.5 ±0.05
pNI 201	λ 607 sep^{+2}	89.5 ±0.05

1. % input hybridizedis(cpm hybridized to excess DNA from named source-cpm hybridized to DNA or blank filters)÷input cpm)
2. The palsmid was amplified by incubation of the strain in chloroamphenicol, removal of the chloroamphenicol, and pulse-labelling.

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such as pBEU 2 (Uhlin *et al.*, 1979). pBEU 2 has 2000 copy numbers per cell at 42°C.

If large amount of PBP-3 can be produced by the strain newly constructed, the PBP-3 will be purified, and the availability of pure PBP-3 will make possible biochemical studies on the protein and the development of a radioimmue assay (RIA). The RIA will replace the Spratt's assay (which require over 30 days for fluorography) and will make possible physiological studies of *sep* function and on regualtion of *sep* translation. Furthermore, the trial of maximizing the gene expression could be applied to other genetic engineering studies.

摘 要

세포분열에 관여하는 유전자중의 하나인 유전자 sep-Penciillin binding protein 3의 유전자-를 λ 607 sep^{+2}로부터 Plasmid pBR 322에 재조합시켰다. 또한 *sep* 유전자의 발현을 최대화하기 위해서 *lac* UV 5와 같은 강한 promoter를 갖고 있는 plasmid pLJ 3에 *sep* 유전자를 재조합시켰으며, *sep* 유전자는 lactose promotor 발현에 적절한 방향으로 위치함을 확인하였다. 새로 재조합된 plasmid 들의 *sep* 유전자 발현도를 조사하기 위해서 이들 plasmids를 포함하고 있는 세포내에서의 se*p* mRNA의 합성량이 측정되었다. *sep* mRNA의 합성량은 *sep* 유전자가 pBR 322내에 있을때, plasmid가 없는 wild type *E. coli* C 600에 비해 약 25배가 증가하였고, *Sep* 유전자가 pLJ 3에 있을때, pBR 322내에 있을때보다 약 50배가 증가하였다.

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