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유전자 재조합 대장균을 사용한 Alpha-interferon의 생산과 분비: 제 1 부. 발현벡터의 제작

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Extracellular Production of Alpha-Interferon by Recombinant *Escherichia coli* : Part I. Construction of Expression Vectors

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ABSTRACT

We constructed hybrid plasmids to allow controlled and extracellular production of human alpha-interferon in *Escherichia coli*. The hybrid plassmids were constructed by transferring alpha-IFN gene from plasmid Hif-2h which has the alpha-IFN gene at PstI restriction site of pBR322, to plasmids pIN-IIIB3 and pIN-IIIC3 at restriction sites between HindIII and BamHI.

Plasmids pIN-IIIB3 and pIN-IIIC3 carry *E. coli* lipoprotein promoter, *lac* promoter and operator in tandem. The plasmids also have *lacI* genes which encode for *lac* repressors, which allows controlled expression of genes cloned to the plasmids by using of inducer IPTG. Lipoprotein signal sequence is located just ahead of cloning sites of the plasmids, which helps cells to excrete or secrete cloned gene products.

Plasmid pUC9 was used as a intermediate vector for transferring of alpha-IFN gene from Hif-2h to pIN vectors in order to solve the problem of different restriction sites between Hif-2h and pIN vectors.

INTRODUCTION

Although all bacterial protein synthesis occurs in the cytoplasm, some proteins are destined to assume extracytoplasmic locatons in the cell envelope or in the environment external to cell. Such proteins are referred to as exported or secreted proteins(1, 2).

E. coli host-vector system for the production of many pharmaceutical proteins, which include interferon, insulin and human growth hormone, raised some problems. One of these problems is the stability of proteins accumulated in the cytoplasm caused by proteolytic degradation. If cloned gene products could be secreted or excreted, the proteins are removed from accessibility to protease, which means possible improvements in recovery. Also the potential physical limitation of intracellular space for accumulation of product could be overcome, there by enhancing yield.

For these purpose we used plasmid vectors containing *E. coli* lipoprotein signal sequence which is reported to play an important role for the extracellular production of proteins. Using plasmids pIN-IIIB3 and pIN-IIIC3 we constructed hybrid plasmid pIF-Ⅲ-B and pIF-Ⅲ-C for the extracellular production of human alpha-IFN.

MATERIALS AND METHODS

Bacterial strains and plasmids, and culture conditions

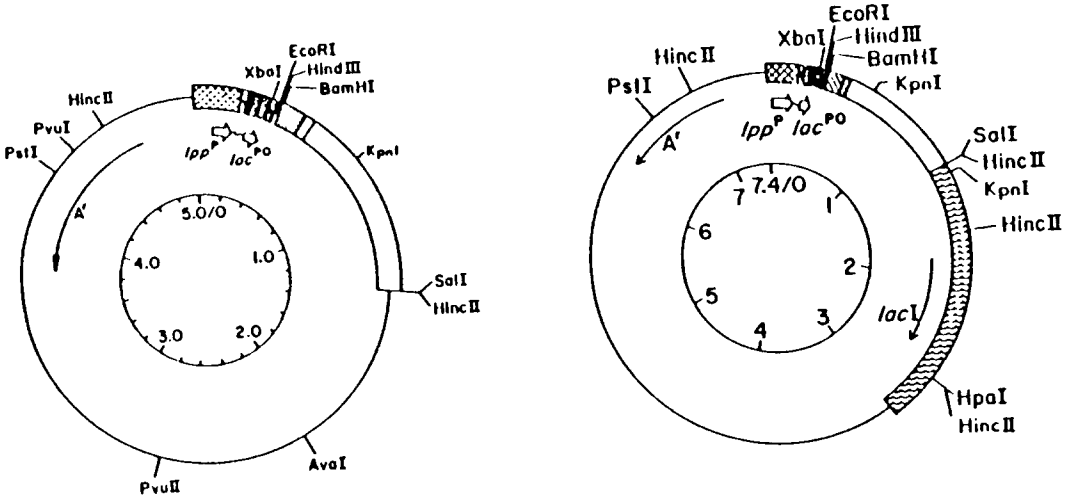
E. coli HB101 (*ara*−14, *proA*−2, *lacY*−1, *galK*−2, *rpsL*20, *xyl*) and JM107 (*supE* 44, *relA1*, *lac-proAB*, *traD* 36, *proAB*, *lacI*^{q}*ZM*15) were used as host strains for plasmids Hif-2h and pUC9, respectively. JE5505 (F , *lpp*-2, *pps*, *his*, *proA*, *argE*, *thi*, *gal*, *lac*, *xyl*, *mtl*, *tsl*)was used as a host strain for transformation of final expression vectors.

All the plasmids used in this study are presented in Table 1. Plasmids pIN-IIIB3 and pIN-IIIC3 which were generously provided by Masayori Inouye(State Univ. of NY at Stony Brook) are shown in Fig. 1. Plasmid Hif-2h was obtained from Chares Weissman.

All the *E. coli* strains were grown overnight at 37℃ in LB broth containing appropriate concentration of antibiotics for the isolation of plasmids. Plasmid-amplification with chlorampenicol was omitted.

Table 1. ***E. coli*** plasmids and their characteristics

Plasmid	Drug marker	Size(kb)	Reference
Hif-2h	Tc^r	5.27	3
pIN-IIIB3	Amp^r	7.40	4
pIN-IIIC3	Amp^r	7.40	4
pUC9	Amp^r	2.70	5



(A) PIN II (B) PIN III

Fig. 1. Structure of pIN vectors.

Plasmid DNA preparations

Plasmids were prepared as described by Kieser(6) with some modifications. The plasmids were pure enough to treat with restriction enzymes and T_4DNA ligase.

Transformations

Transformation of *E. coli* strains with plasmid DNAs or cross ligated DNAs was performed as described by D.A. Morrison(7). Transformants were screened on LB plate with an appropriate antibiotic, 0.8% soft agar supplemented with 1mM IPTG and 40㎍/ml of X gal was used for the detection of insertionally inactivated β-galactosidase gene of pUC9.

DNA manipulations

Restriction endonucleases were obtained from Bethesda Research Laboratories and T4 DNA ligase was purchased from New England Biolabs. Digestion and ligation of DNAs were performed by following the instructions of the enzyme suppliers. Separation of required DNA fragments was performed by electroelution of the DNA fragments onto diethyl aminoethyl cellulose paper(8) during agarose gel electrophoresis.

RESULTS AND DISCUSSION

Construction of intermediate vector pIF908

We considered to transfer alpha-IFN gene from plasmid Hif-2h to pIN-III vectors. Overall schematic diagram is shown in Fig. 2. Because pIN vectors have no PstI site,

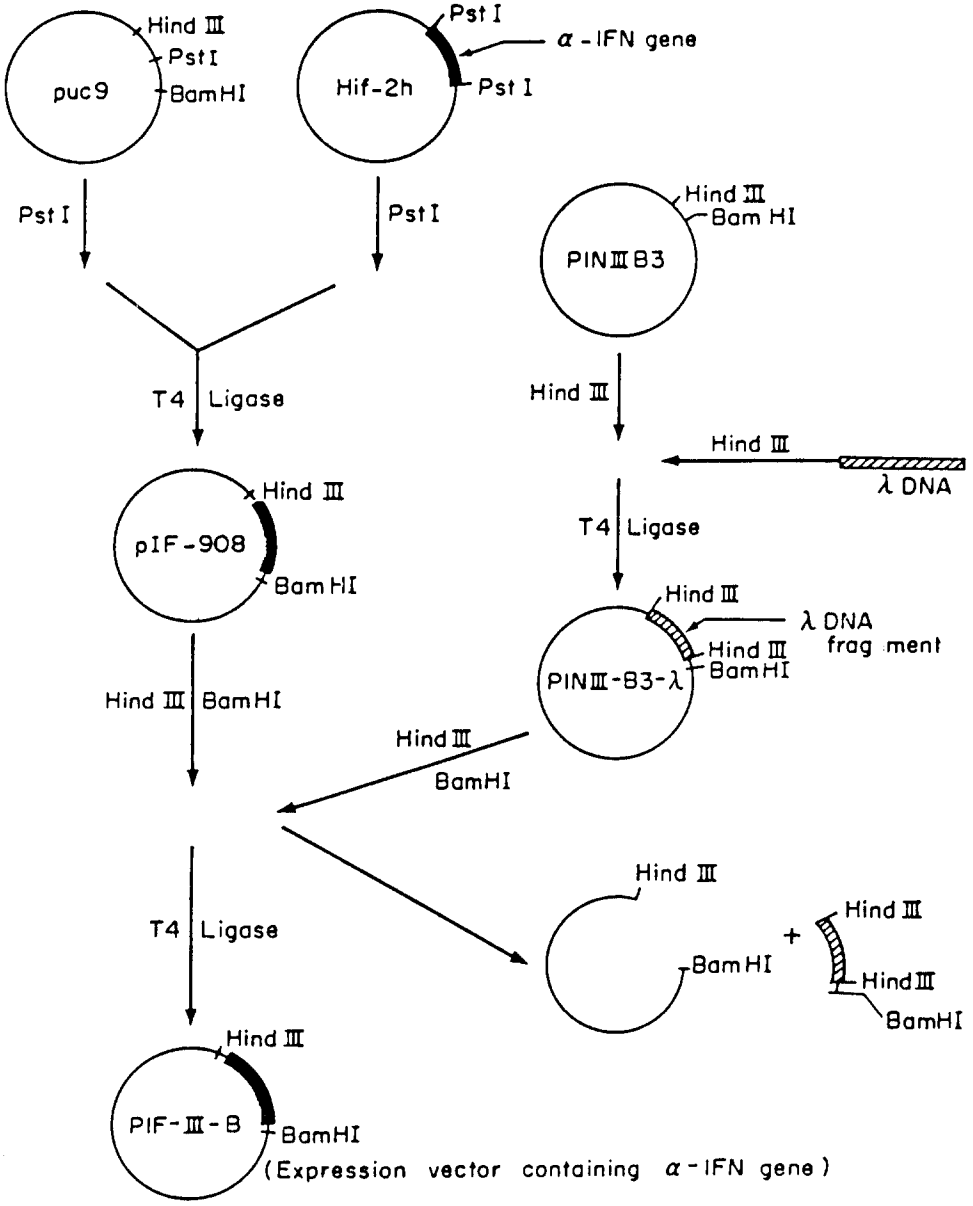


Fig. 2. Overall schematic diagram of the transfer of α-IFN gene from vectors to vectors.

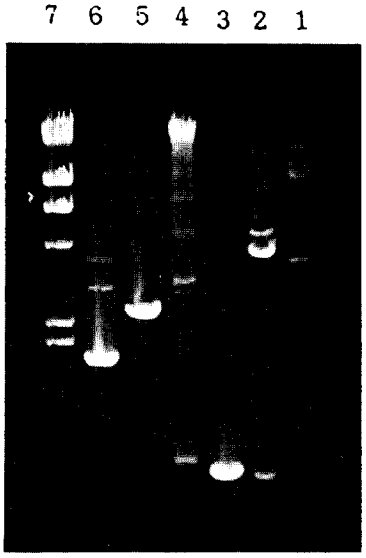
plasmid pUC9 was used as an intermediate vector for transferring alpha-IFN gene between two plasmids.

The pUC vectors, which were developed by Messing (8) on the base of the M13m series, contain a *lac* promoter, a *lac* operator, and a *lacZ* gene in the middle of which multiple cloning site exists. Therefore, when a foreign gene is inserted into the multiple cloning site and then expressed by an inducer, IPTG the *lacZ* protein, *i. e.* β-galactosidase which can degrade X-gal, is not synthesized because it is insertionally inactivated.

Only 0.9lkb size of alpha-IFN gene fragments were recovered from PstI digests of Hif-2h and ligated with the PstI-digested pUC9. Electrophoretic diagram for the work is shown in Fig. 3. The ligates were used to transform *E. coli* strain JM107. The scheme is shown in Fig. 4. White colonies having alpha-IFN gene-cloned pUC9 were screened by overlay of soft agar supplemented with IPTG and X-gal on LB agar containing 50μg/ml of ampicillin. Ligation orientations of the hybrid plasmids were checked by EcoRI digestion of the plasmids and comparing the size of DNA fragments through the agarose gel electrophoreses. Hybrid plasmid carrying alpha-IFN gene with required ligation-orientation was named pIF908. Pictures for the orientation check and electrophoresis are shown in Fig. 5.

Construction of final expression vectors

Plasmid pIN-IIIB3, which carries a lipoprotein promoter of *E. coli*, a *lac* promoter, a *lac* operator and a lipoprotein signal sequence in tandem, has cloning sites for EcoRI, HindIII and BamHI at the end of *lpp* signal sequence. The cloning sites are too close to be treated with more than two restriction enzymes. HindIII-cleaved fragment of bacteriophage lambda DNA was inserted into the HindIII site of pIN-IIIB3 in order to lengthen the distance of cloning sites between HindIII and BamHI. The hybrid plasmid was named pIN-IIIB3-λ. The procedure for the construction of the hybrid plasmid and electrophoresis picture are shown in Fig. 6. HindIII and BamHI double digested insert DNAs of alpha-IFN gene from pIF908 were



1. Hif-2h
2. Hif-2h : PstI
3. α-IFN gene band taken from DEAE paper
4. Ligate
5. pUC9 : PstI
6. pUC9
7. Lambda DNA : HindIII

Fig. 3. Electrophorogram for the construction of plasmid pIF908.

ligated with HindIII and BamHI double digested DNAs of pIN-IIIB3-λ. *E. coli* strains JE5505 which are mutants unable to produce lipoprotein were transformed by the ligates. And transformants were selected on the agar plates containing 50㎍/ml of ampicillin. The final expression vector was named pIF-III-B. The scheme and electrophoretic picture for the work are presented in Fig. 7. Another expression vector pIF-III-C was also constructed using pIN-IIIC3 instead of pIN-IIIB3 via exactly same procedure as in pIF-III-B. The only difference expected between pIF-III-B and pIF-III-C lies in that the former retains some of its gene products in the periplasmic space in free form while the latter in covalent esther linkage with lipid. The structure of the plasmids, pIF-III-B and pIF-III-C are shown in Fig. 8.

There are some problems in producing eukaryotic proteins in bacterial host strains via recombinant DNA techniques. One is the stability of gene product accumulated in cytoplasm of the cell due to proteolysis. Another is the detrimental effect of overproduced gene products on host cell physiology. Overproduction of some foreign gene product and their accumulation in cytoplasm also cause to form inclusion body *i. e.* protein aggregates which are not as active as native proteins(9).

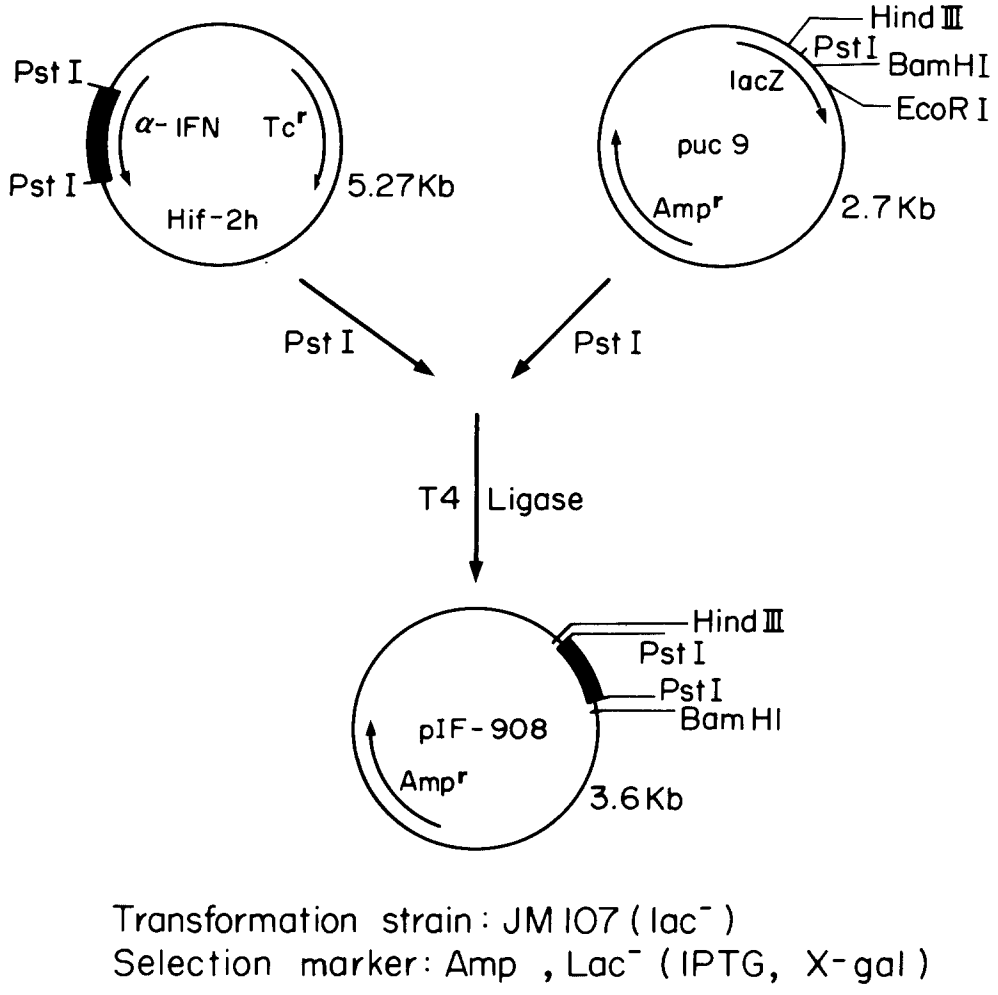
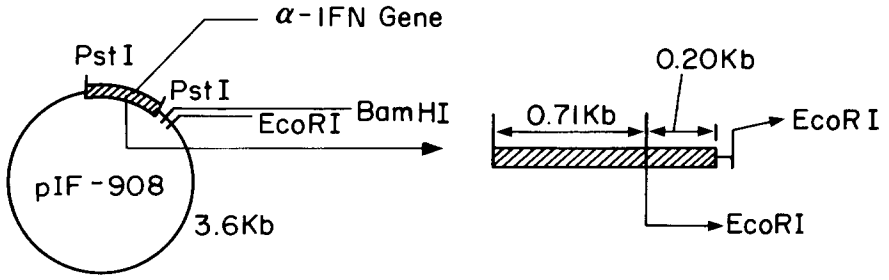


Fig. 4. Transfer diagram of α-IFN gene from Hif-2h to intermediate vector puc9.

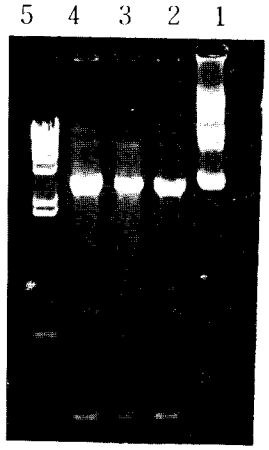
If the proteins could be exported to external environment from the cytoplasm, these problems would not be so serious. And if excretion of proteins is very efficient one need not disrupt the cells to recover the products, saving efforts to separate the target products from other intracellular proteins and chromosomal DNAs. We employed vector systems capable of transferring gene product to extracellular environment and controllable of expression by using inducer, IPTG in order to solve these problems.

Now that we finished the construction of hybrid plasmids, pIF-III-B and pIF III-C, we expect the plasmids to produce alpha-IFNs with enhanced yields via optimization of environmental conditions including induction point.

Lipoprotein of *E. coli* is thought to be one of the most abundant proteins in terms of number of molecules in the periplasm implying that its promoter is very strong. And expression of alpha IFN gene in plasmids pIF-III-B and pIF III-C is expected to be very efficient under *lpp* promoter.

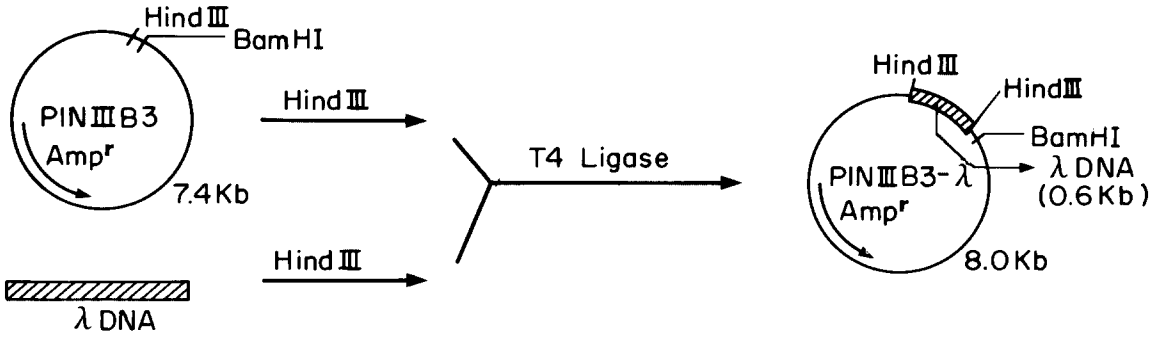


If orientation is correct, when pIF-908 is digested with EcoRI, two fragements of 3.4Kb and 0.20Kb are produced.



1. pIF-908
2. pIF-908 : EcoRI
3. "
4. "
5. Lambda DNA : HindIII

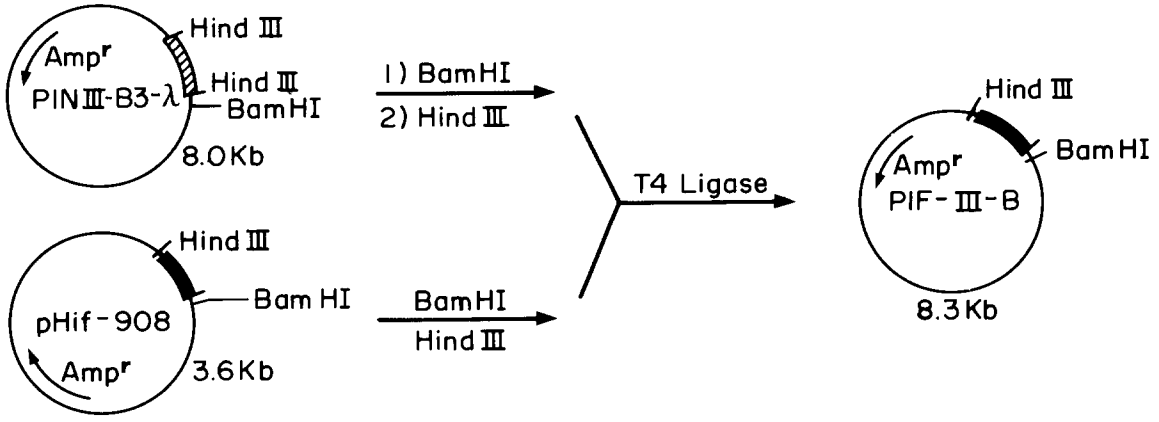
Fig. 5. Electrophorogram for the orientation check of the alpha-IFN gene in the plasmid of pIF-908.

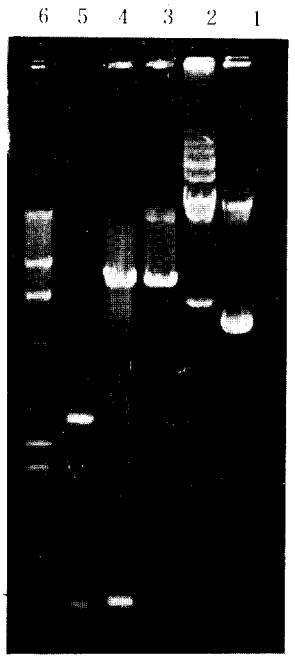


Transformation Strain: JE5505
Selection Marker: Amp^r

1. pIN-III B3
2. pIN-III B3 : Hind III
3. pIN-III B3-λ
4. pIN-III B3-λ : Hind III
5. Lambda DNA : Hind III

Fig. 6. The construction of pIN-Ⅲ B3-λ.





1. pIN-III B3
2. pIF-III-B
3. pIN-III B3: BamHI
4. pIF-III-B: HindIII, BamHI
5. pIF-908: PstI
6. Lambda DNA: HindIII

Fig. 7. Electrophorogram for the construction of the last expression vector pIF-III-B.

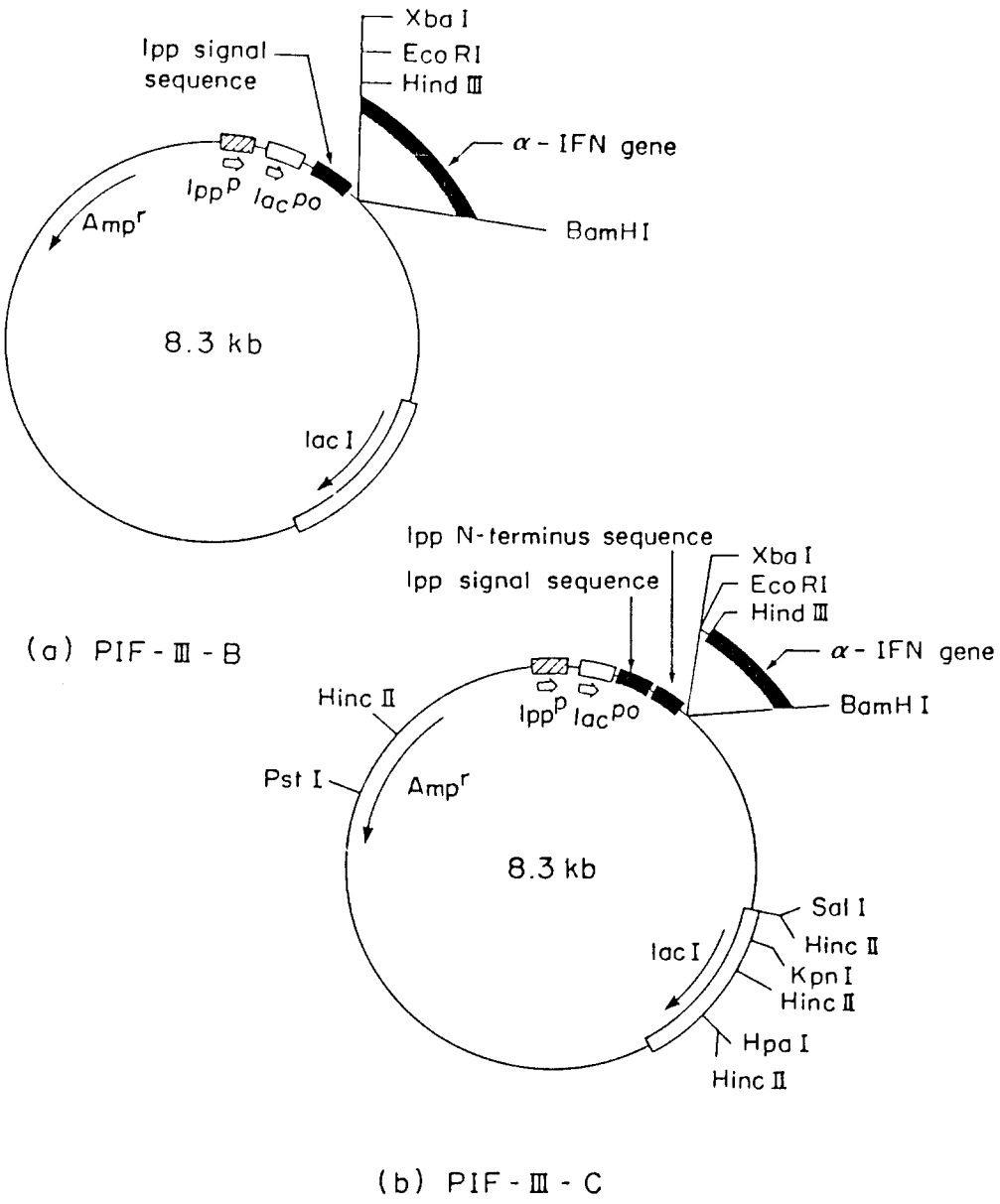


Fig. 8. Structure of the final alpha-IFN expression vectors pIF-III-B and pIF-III-C made from pIN-III B3 and pIN-III C3, respectively.

ABBREVIATIONS

Amp^r: ampicillin resistant, IFN: interferon, IPTG: isopropyl-β-D thiogalactopyranoside, kb: kilo base pairs, *lac*: lactose, LB: Luria Bertani, *lpp*: lipoprotein, Tc^r: tetracycline resistant,

X-gal: 5-bromo-4-chloro-3-indolyl β-D-galactoside

요 약

대장균으로부터 alpha interferon의 생산과 분비를 유도하기 위해 대장균의 lipoprotein promoter, lactose promoter 및 operator와 lipoprotein의 signal sequence를 가지는 vector 에 alpha-IFN 유전자를 cloning하여 발현 vector pIF-III-B와 pIF-III-C를 제작하였다.

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